Universidade de Aveiro Departamento de Biologia
2003

Ana Gabriela da Silva Cavaleiro Henriques

O efeito do stress celular na proteína precursora de amilóide de Alzheimer

The effect of cellular stress on the Alzheimer´s amyloid precursor protein

Universidade de Aveiro Departamento de Biologia
2003

Ana Gabriela da Silva Cavaleiro Henriques

O efeito do stress celular na proteína precursora de amilóide de Alzheimer

The effect of cellular stress on the Alzheimer´s amyloid precursor protein

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia Molecular, realizada sob a orientação científica da Profª. Doutora Odete da Cruz e Silva, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro e co-orientação do Prof. Doutor Edgar F. da Cruz e Silva, Professor Associado do Departamento de Biologia da Universidade de Aveiro.

o júri

presidente

Professor Dr. Edgar F. da Cruz e Silva
Professor Associado da Universidade de Aveiro

Professor Dr. António Correia
Professor Auxiliar da Universidade de Aveiro

Professora Dr.ª Odete A. B. da Cruz e Silva
Professora Auxiliar da Universidade de Aveiro

Professora Dr.ª Margarida D. Amaral
Professora Auxiliar da Universidade de Lisboa

acknowledgments

To Prof. Dr. Odete da Cruz e Silva for her guidance and help during the dissertation of my thesis

To Prof. Dr. Edgar da Cruz e Silva for the light moments when necessary

To FCT, in particular project POCTI 33520/99 entitled “Stress and AD: a molecular cellular approach”, and to the CBC

To all my laboratory colleagues for their support and for their comradeship

To all my friends for their unconditional friendship

To my grandparents, Amélia and Carlos for all the help

To my parents, Graça and António Manuel, and my brother, Ricardo, for being there when I needed

To André for his never ending care and patience at home

resumo

A proteína precursora de amilóide de Alzheimer (APP) é um factor chave na doença de Alzheimer (AD). Essencialmente o processamento da APP resulta na produção de Abeta, o peptídeo tóxico depositado nas placas de amilóide dos indivíduos com AD. Ainda permanece por esclarecer se o processamento da APP é afectado sob condições de stress celular, potencialmente aumentando a quantidade de Abeta produzida. Além disso, o stress celular pode induzir alterações moleculares, associadas à AD, que podem representar marcadores moleculares úteis para o diagnóstico da AD.

Com estas questões em mente, procurámos identificar alterações, em resposta ao stress celular, no processamento da APP e na expressão de outras proteínas. Nestes estudos de monitorização considerámos que a fosforilação proteica anormal e o stress oxidativo podem contribuir para a condição patológica. Assim, investigámos o processamento da APP dependente da fosforilação durante o stress celular. Os dados obtidos confirmam que a secreção da APP é reduzida em situações de stress, e que o efeito é idêntico em linhas celulares de tipo neuronal e não neuronal. Os resultados obtidos revelam que o PMA, mesmo em situações de stress (azida de sódio 1 mM) pode afectar o processamento da APP, aumentando a produção de sAPP (o fragmento secretado após o processamento de APP) que pode potencialmente reduzir a produção de Abeta. A hipótese de afectar a produção de Abeta dependente da fosforilação, que por sua vez pode ter relevância num quadro clínico, mantém-se mesmo em condições de stress.

Os resultados revelaram que a indução de sAPP, após a adição de ésteres de forbol, ou ácido ocadeíco, em condições de stress não é idêntica. Em contraste, sob condições controlo, tanto os ésteres de forbol como o ácido ocadeíco produzem o mesmo efeito em termos da produção de sAPP. Aparentemente estas duas vias podem ser dissociadas em condições de stress, o que de algum modo pode reflectir processamento alterado da APP em condições adversas.

Nas experiências em que se analisou a expressão de outros potenciais marcadores moleculares, foram detectadas alterações nos níveis de expressão de várias proteínas. Estes marcadores moleculares representam alvos interessantes para futura validação e potenciais candidatos para um diagnóstico molecular na AD. As proteínas já identificadas são importantes do ponto de vista da transdução de sinais, e incluem a PP1, a HSP70, a PARP e a própria APP.

abstract

The Alzheimer's amyloid precursor protein (APP) is a key factor in Alzheimer's disease (AD). Essentially the processing of APP results in the production of Abeta, a toxic peptide found deposited in the amyloid plaques of individuals with AD. It is unclear whether under conditions of cellular stress APP processing is affected, potentially increasing the amount of Abeta being produced. Further the molecular alterations induced by cellular stress, and associated with AD, could present attractive molecular markers for the diagnosis of AD.

Bearing these questions in mind we set out to identify cellular stress-related alterations in APP processing and altered expression of APP and other proteins. When monitoring potential molecular markers we had in mind several findings describing that both abnormal protein phosphorylation and oxidative stress can contribute to the disease condition. Thus, we investigated phosphorylation-dependent APP processing during cellular stress. The data confirmed that APP secretion is reduced under stress, and that the effect is similar in both neuronal like and non-neuronal cell lines. The present results reveal that PMA, even under stress conditions (1 mM sodium azide), can affect APP processing, increasing sAPP (a fragment secreted following the processing of APP) production which can potentially reduce Abeta production. The hypothesis whereby affecting Abeta production in a phosphorylation-dependent manner and that this may be of clinical relevance appears to hold true even under stress conditions.

The results also revealed that induction of sAPP upon addition of phorbol esters, or okadaic acid, under conditions of stress was not the same. This contrasts markedly with control conditions where both phorbol esters and okadaic acid produce the same effect in terms of inducing sAPP production. Hence, it appears that these pathways can be dissociated under conditions of stress, which may in fact reflect altered APP processing during adverse conditions.

In the experiments addressing the expression levels of other potential molecular markers, alterations in apparent expression levels of several proteins could be detected. These molecular markers provide interesting targets for further validation and potential candidates for a future molecular diagnosis of AD. Proteins thus far identified are important from a signal transduction point of view and they include, protein phosphatase1, HSP70, PARP and APP itself.

INDEX

ABBREVIATIONS

AD	Alzheimer´s Disease
APP	Alzheimer´s amyloid precursor protein
ApoE	Apolipoprotein E
ATP	Adenosine 5´- triphosphate
BACE	?-site APP cleaving enzyme
BCA	Bicinchonic acid
BCIP	Bromocloroindolyl phosphate
BSA	Bovine serum albumin
CNS	Central nervous system
DMEM	Dulbecco's modified Eagle's medium
2DG	2-deoxyglucose
ECL	Enhanced chemiluminescence
FAD	Familial Alzheimer´s Disease
G_o	G protein
HSPs	Heat shock proteins
iNOS	Inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
KPI	Kunitz-type serine protease inhibitor
MTT	[3-(4,5dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]
MW	Molecular weight
NAD	Nicotinamide adenine dinucleotide
NBT	Nitro blue tetrazolium
NFT	Neurofibrillary tangles
OA	Okadaic acid
PARP	Poly (ADP-ribose) polymerase
PCD	Programmed cell death
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PP2B	Protein phosphatase 2B
PS1	Presenilin 1
PS2	Presenilin 2

RIP	Regulated intremembrane proteolysis
ROS	Reactive oxygen species
sAPP	Secreted Alzheimer´s amyloid precursor protein
SAPK	Stress activated protein kinase
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
TACE	Tumor necrosis factor-? converting enzyme
TBS	Tris buffered saline
TBS-T	Tris buffered saline-tween
TNF-?	Tumor necrosis factor-?

1. INTRODUCTION

1.1 Alzheimer´s Disease

Alzheimer´s disease (AD), first described by the German pathologist Alois Alzheimer in 1907 (Alzheimer, 1907), is presently the most common neurodegenerative disease among the eldery. This disorder of the central nervous system (CNS) can occur in people in their 40s and 50s, but it most often affects those in their 60s and older. AD is a form of dementia that invariably leads to a complete loss of all cognitive abilities and premature death. Individuals affected by AD exhibit a progressive deterioration of memory and logical thinking. In the later stages perception and orientation are affected as well as loss of personality and intellect to an extent that interferes with daily activities.

1.1.1 Neuropathological hallmarks of Alzheimer´s Disease

Prominent histopathological features of this disease include the extracellular deposition of insoluble amyloid deposits – *senile plaques* – in the brains of affected individuals and also the accumulation of intracellular *neurofibrillary tangles* (NFT) (Grundke-Iqbal *et al.*, 1986; Goedert *et al.*, 1992) (Figure 1). NFT contain aggregates of proteins, mainly composed of hyperphosphorylated tau protein. In AD, the latter is present as insoluble filaments, usually paired helical filaments. Tau is a microtubule-associated protein which is normally quite soluble and the cellular events that result in filament formation are not well understood. A key normal function of tau is to promote the bundling of neurofilaments in axonal processes of neurons. The extent to which tau promotes this activity depends on its phosphorylation. Hence, changes in the activation of signalling cascades leading to abnormal phosphorylation or aberrant tau aggregation can potentially contribute to the formation of NFT resulting in neuronal degeneration.

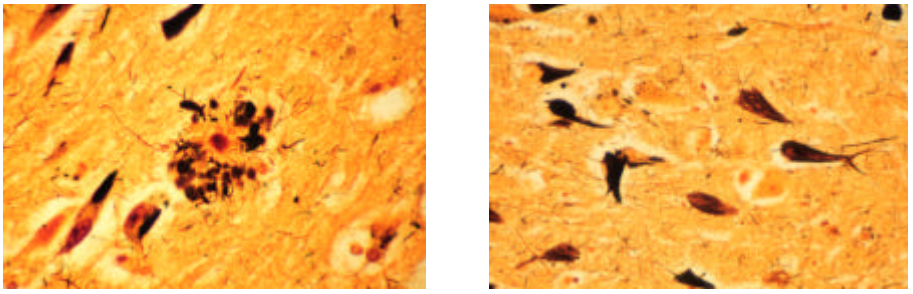

Figure 1. Hallmarks of Alzheimer´s Disease. Left Panel: Silver stain of a senile plaque. The major component of the plaque is the amyloid Abeta peptide. Right Panel: silver stain shows dark–staining NFT in individual neurons. In AD tau becomes hyperphosphorylated changing the structure of neurofilaments. Although the senile plaques and the NFT are pathological hallmarks of AD, many elderly persons have small numbers of these structures in their brains without manifesting the disease. (Taken from www.medsch.wisc.edu/path703/slide/lectslides/cnsdegen.html).

As already mentioned, another neuropathological hallmark of the disease is the presence of *senile plaques* in the brain. These extracellular deposits of fibrillar aggregates are mainly composed of a 4-KDa peptide termed beta-amyloid (Abeta) (Glenner and Wong, 1984). Abeta is a proteolytic fragment derived from the Alzheimer´s amyloid precursor protein (APP) and although Abeta exists as an aggregated, poorly soluble form in brain deposits, it is secreted from cells during normal metabolism as a soluble molecule (Haass *et al.*, 1992; Shoji *et al.*, 1992).

The principals underlying cellular features of AD are the degeneration and eventual loss of neuronal cells. This extensive degeneration affects many types of neurons and may account for the numerous neurological deficits shown by affected individuals. Neurodegeneration is most pronounced in the hippocampus, cerebral cortex and amygdala (Price *et al.*, 1993; Williams, 2001), regions of the brain that play a major role in memory, cognition and behaviour. However, the relationship and all the sequential factors that result in NFT, *senile plaques*, and finally in AD have not been fully described or understood. Nonetheless it is clear that AD is a multifactorial disorder which includes genetic risk factors, abnormal protein processing and external factors such as cellular stress.

1.1.2 Genetic risks for Alzheimer Disease

Approximately 95% of AD cases are sporadic and appear at a late age, while the remaining 5% of cases have an autossomal dominant pattern of inheritance. The AD cases of genetic etiology are termed familial Alzheimer´s disease (FAD). Familial history of dementia is a risk factor in AD but several other aspects may influence the development of the disease (Richard and Amouyel, 2001). Although only a small portion of AD cases have a genetic basis, the molecular and genetic study of these familial cases allows for increased knowledge about the etiology of the more abundant sporadic forms, since increased Abeta production and accumulation is a common feature of both.

Genetic analyses have led to the identification of three "causative" genes for early-onset FAD (? 60 years): APP on chromosome 21, presenilin 1 (PS1) on chromosome 14, and presenilin 2 (PS2) on chromosome 1.

Within the APP gene various locations carrying mutations (Figure 2) have been identified (Chartier-Harlin *et al.*, 1991; Goate *et al.*, 1991; Hardy and Allsop, 1991; Murrell *et al.*, 1991; Mullan *et al.*, 1992). These mutations result in a single amino acid substitution [or a double amino acid substitution (Lys^{595}, Met^{596} ↴ Asn, Leu; see Figure 2), namely in the Swedish mutation]. Significantly, mutations in APP (all lying near or within the Abeta domain) associated with FAD result in APP being more efficiently processed by secretases, thus generating increased amounts of Abeta (Citron *et al.*, 1992, Cai *et al.*, 1993, Haass *et al*., 1995), thereby promoting amyloidogenesis. Furthermore, the level of APP being expressed also appears to be an important aspect. For instance, in Down's Syndrome, caused by trissomy of chromosome 21, there is an extra copy of the APP gene. These individuals show increased levels of Abeta and invariably develop plaques and tangles in their brains, with clinical dementia in many cases before the age of 50 (Tanzi *et al.*, 1987; Selkoe 1997; Esler and Wolfe, 2001).

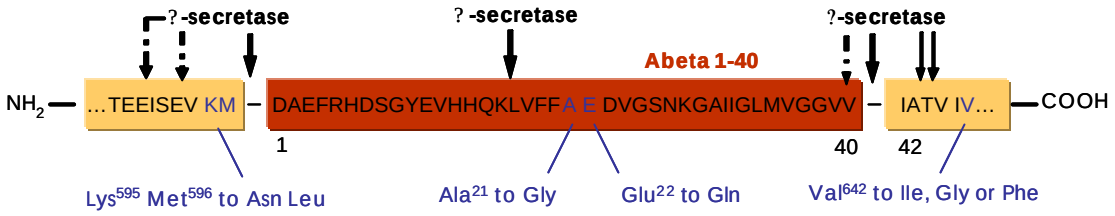

Figure 2. APP mutations lying near or in the Abeta domain. The Figure shows APP with the Abeta domain and the cleavage sites of the secretases involved in APP processing. Residues in blue indicate genetic mutations already described.

Overall, APP mutations are responsible for only a small number of FAD cases (Tanzi *et al.*, 1992). The majority of early-onset cases have been associated with mutations in PS1 and PS2, that are known to regulate APP processing (De Strooper *et al.*, 1998). More importantly, presenilin mutations linked to FAD all increase processing of APP and Abeta formation (Duff *et al.*, 1996; Scheuner *et al.*, 1996; Selkoe, 1997). AD is clearly a multifactorial disorder, where genetic predisposition also plays a relevant role. Apolipoprotein E (ApoE) is an inherited “risk factor” for late-onset FAD (? 60 years) on chromosome 19 (for review see Tanzi *et al.*, 1996). Allelic variation of ApoE is an important contributing factor for sporadic AD cases, since apolipoprotein E binds to Abeta, potentially affecting the formation of senile plaques (Selkoe, 1997; Holtzman, 2001).

In all FAD cases there is evidence for excessive secretion of Abeta and both genetic and sporadic cases exhibit Abeta deposition in brain parenchyma and blood vessels (Iwatsubo *et al.*, 1994; Wang *et al.*, 1999), that invariably leads to the development of AD. This observation, coupled with studies demonstrating that Abeta is neurotoxic to neurons (Yankner *et al.*, 1990; Kowall *et al.*, 1992; Pike *et al.*, 1992; Butterfield, 1997), led to the concept that Abeta is central to the pathogenesis of AD.

1.1.3 Alzheimer´s amyloid precursor protein and Abeta genesis

Abeta arises from the intracellular processing of APP, which is encoded by a gene on chromosome 21 and is constitutively secreted by both neuronal and non-neuronal mammalian cells into the extracellular fluid (Selkoe, 1994). APP is an integral transmembrane glycoprotein containing a membrane-spanning domain (Kang *et al.*, 1987; Tanzi *et al.*, 1987). Several functions for APP and its derivates have been described (Buxbaum and Greengard, 1996; Kosik *et al*., 1996; Selkoe *et al*., 1996), including cell-surface receptor (possible G_O coupled) involved in signal transduction, protease inhibition, cell adhesion molecule, regulation of neuritic outgrowth, promotion of cell survival, protection against a variety of neurotoxic insults, stimulation of synaptogenesis, modulation of synaptic plasticity, and in learning and memory processes (Mattson *et al*., 1997; Dodart *et al*., 2000).

Alternative post-transcriptional splicing of the APP gene produces different isoforms of this protein ranging from 365-770 amino acid residues (Kosik, 1993). The three major isoforms expressed in the brain have 695, 751 and 770 amino acids (APP_{695}, APP_{751} and APP_{770}, respectively). The 751- and 770- amino acid spliced isoforms contain a Kunitz-type serine protease inhibitor (KPI), which may regulate the degradation of APP (Edelberg and Wei, 1996). APP_{695}, the most abundant isoform expressed in neurons, lacks the KPI (Goedert, 1987; LeBlanc *et al.*, 1991). APP is both N- and O-glycosylated (Weidemann *et al.*, 1989) in its mature form while immature APP is N-glycosylated only. Protein glycosylation appears to play a key role in APP processing and function (Pahlson and Spitalnik, 1996).

APP metabolism is complex and can occur via several pathways (Nitsch *et al.*, 1994; Checler, 1995; Selkoe *et al.*, 1996) which have not been fully elucidated. In its simplest form APP processing can be described as occurring via two major pathways, one being non-amyloidogenic and the other amyloidogenic (Figure 3). In the non-amyloidogenic (or ?-secretase) pathway, the ?-secretase enzyme cleaves APP within the Abeta domain and therefore prevents its production. The produced soluble N-terminal APP fragment is termed sAPP? and further degradation of the C-terminal fragment by a ?-secretase, gives rise to a small fragment termed p3. The amyloidogenic (or ?-secretase) pathway involves a ?-secretase enzyme which cleaves APP N-terminally to Abeta, and a subsequent ?-secretase cleavage liberating Abeta peptides of 40 or 42 amino acids that can

aggregate and form senile plaques. The latter pathway also generates a soluble APP N-terminal fragment termed sAPP? (Figure 3). However, this classification scheme appears to be an oversimplification, as N-terminal truncated Abeta peptides such as p3 have been isolated from early-stage diffuse plaques and form amyloid fibrils even more readily than full-length Abeta (Gowing *et al*., 1994; Pike *et al*., 1995).

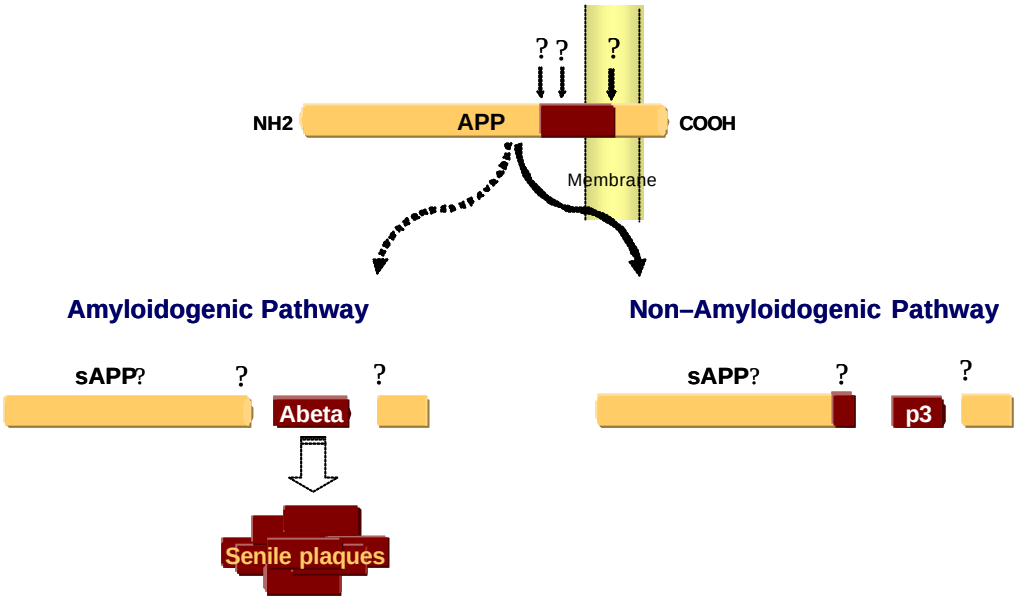

Figure 3. APP Processing. APP can be processed by two major pathways: the amyloidogenic pathway that gives rise to the toxic Abeta fragment, which can aggregate and form senile plaques, and the non-amyloidogenic pathway, which releases a small peptide known as p3.

Moreover, considerable work has been dedicated to identifying all the secretases involved in these pathways. Likely candidates include two ezymes in a disintegrin and metalloprotease (ADAM) family: ADAM10 and ADAM-12 [or TACE ? tumor necrosis factor-? (TNF-?) converting enzyme] having either inherent ?-secretase activity or are thought to be somehow involved in the regulation of ?-secretase (Buxbaum *et al*., 1998; Sabo *et al*., 1999).

BACE1 (?-site APP-cleaving enzyme) also denominated Asp2 or memapsin 2 has been identified as a ?-secretase, since it demonstrates all the properties of this enzyme, being responsible for the first step in the process that ultimately result in Abeta formation (Vassar *et al*., 1999). Moreover, intracellular localization of BACE1 is consistent with the generation site of Abeta.

1.1.4 A new avenue for neuronal signalling transduction in Alzheimer's Disease

Recent evidence from several groups suggests that the generation of signalling proteins by "regulated intramembrane proteolysis" (RIP) is a new paradigm of signal transduction (Ebinu and Yankner, 2002). RIP occurs when a transmembrane protein is cleaved within the transmembrane domain, releasing a cytoplasmic fragment that can act directly in the nucleus to activate gene expression. This occurs in two steps. Cleavage of the protein outside the membrane (usually in response to ligand binding) results in a conformational change, which in turn triggers a second intramembrane cleavage event that releases an active cytoplasmic fragment. The latter translocates to the nucleus and activates gene expression (Ebinu and Yankner, 2002).

The discovery that APP is one of the mammalian proteins that may be processed by RIP is extremely interesting and opens new avenues towards our understanding of the diverse signal transduction pathways (Selkoe and Kopan, 2003). Intramembranous gamma-secretase cleavage of APP plays a central role in the formation of Abeta peptide. An intriguing possibility is that the cognate C-terminal fragment generated by gamma-secretase cleavage could also play a role through the regulation of nuclear signalling events. Thus, RIP may contribute to both brain development and degeneration and may provide unexpected diversity to the signalling repertoire of a cell (Ebinu and Yankner, 2002).

1.2 Protein phosphorylation and Alzheimer´s Disease

1.2.1 Phosphorylation dependent APP metabolism

Of particular interest is the observation that APP metabolism via different pathways is phosphorylation dependent. Accordingly, several groups have shown that sAPP release into cell medium can be increased upon addition of phorbol esters. Although the factors affecting APP metabolism/processing have not been fully elucidated, it is now widely accepted that phosphorylation of APP itself could play an important role. Although this has yet to be clearly demonstrated, several kinases and phosphatases have been described as key players relevant to APP processing. A strong case has been made for protein kinase C (PKC) in the regulation of APP processing both *in vitro* (Nitsch and Growdon, 1994) and *in vivo* (Caputi *et al.*, 1997; Savage *et al.*, 1998). Direct activation of PKC by phorbol esters has been shown to regulate APP cleavage in several cell lines (Buxbaum *et al.*, 1990; Gabuzda *et al.*, 1993; Jacobsen *et al.*, 1994; Nitsch *et al.*, 1996; Mills *et al.*, 1997; Mills and Reiner, 1999). *In vitro*, the levels of Abeta ?produced can be decreased and the amounts of sAPP produced via the ?-secreatase pathway increased upon the addition of PDBu (Buxbaum *et al*, 1993; Caporaso *et al*, 1992). Observations that APP could be phosphorylated both *in vitro* (Gandy *et al*, 1988, Suzuki *et al*, 1994) and *in vivo* in rat brain and several cell lines (Oishi *et al.*, 1997) on the cytoplasmic domain on residues Thr^{654} and Ser^{655}, made it a strong candidate as a substrate for the above mentioned activity. However, it is known that PDBu-stimulated release of sAPP can occur independently of the phosphorylation state of APP. Indeed, direct phosphorylation of APP by PKC is not required because the deletion of the cytoplasmic tail of APP did not inhibit the increase in the release of sAPP elicited by phorbol esters (da Cruz e Silva *et al.*, 1993; Hung and Selkoe, 1994). Hence, the action of PKC, promoting ?-secretase cleavage has not been fully elucidated.

1.2.2 Protein phosphatases in Alzheimer´s Disease

Protein phosphatases are also important in APP processing and in the phosphorylation of Tau protein. The three most common types of Ser/Thr-phosphatases are protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A) and protein phosphatase 2B (PP2B). PP2A, PP2B, and to a less extent, PP1 can all dephosphorylate Tau *in vitro* (Gong *et al.*, 2000).

PP1, the most widely expressed and abundant protein Ser/Thr-phosphatase, is likely to play a central role in the production of sAPP, since the half-maximal dose for stimulation of APP secretion was approximately 100-fold higher with okadaic acid (OA, an inhibitor of serine/threonine specific protein phosphatases) than with calyculin A (da Cruz e Silva *et al.*, 1995a). Similarly, exposing a rat brain cell line to Abeta, Tan *et al*. (1997), showed that one 50-60 KDa protein became more phosphorylated. Furthermore, using several kinase and phosphatase inhibitors they could block both increase phosphorylation of this protein and Abeta toxicity. Additionally, Abeta also causes a general increase in overall phosphatase activity. It is therefore likely that a protein phosphorylation cascade is involved in Abeta toxicity (Tan *et al.*, 1997).

Moreover, PP1 is known to be highly expressed in adult mammalian brain both in neurons and glia (da Cruz e Silva *et al.*, 1995b; Ouimet *et al.*, 1995). Although ubiquitously expressed throughout the periphery, the three known PP1 catalytic subunits (PP1alpha, PP1beta and PP1gamma) are particularly abundant in mammalian brain. PP1 has been linked to the efficacy of learning and memory by limiting the acquisition of new knowledge and favouring memory decline (Genoux *et al.*, 2002). PP1 inhibition prolongs memory when induced after learning, suggesting that PP1 promotes "forgetting". These findings may account for age-related cognitive decline and emphasize the physiological importance of PP1 as a suppressor of learning and memory. Thus, at least in mice, the molecular machinery is not completely deteriorated with aging and the results show that near normal cognitive functions can be restored by simply inhibiting PP1. It would appear therefore that altered PP1 activity may be associated with normal cognitive decline during aging and represents an important molecule to monitor in AD related studies.

1.3 Oxidative stress associated events in Alzheimer´s Disease

As previously mentioned, AD is a multifactorial disease, at the centre of which lies a cascade of biochemical events which can contribute to the pathogenesis of the disease condition. Oxidative stress is such a contributing factor which has received considerable attention. Indeed, oxidative stress is extensive in the AD brain (Markesbery, 1997, Markesbery and Carney, 1999; Smith *et al.*, 2000), and occurs when the generation of reactive oxygen species (ROS) exceeds intrinsic antioxidant defences of living cells.

There is evidence that oxidative damage, mediated by free radical injury, may be one of the initiating and promoting factors responsible for neurodegeneration in AD (Prasad *et al.*, 2002). Further, oxidative stress precedes plaque and tangle formation, as demonstrated using transgenic animals bearing mutations leading to plaque formation (Pratico *et al.*, 2001, reviewed in Gibson, 2002). Although the initial sources of oxidative stress remain unclear, the process seems highly dependent on the four aspects described below:

1) Active microglia around senile plaques. An oxidative inflammatory response can be initiated due to the release of reactive oxygen intermediates, nitric oxide and inflammatory cytokines by activated microglia triggered by Abeta deposits (Colton and Gilbert, 1987; Lukiw and Bazan, 2000; Butterfield *et al*., 2001).

2) Redox-activity of transition metals such as iron and copper (Perry *et al.*, 2002). The levels of iron (in a redox-active state) are increased in *NFTs* as well as in Abeta deposits (Smith *et al.*, 1997), iron catalyses the production of radical hydroxyl from H_2O_2. Furthermore, aluminium, which also accumulates in *NFT* containing neurons, stimulates iron induced lipid peroxidation.

3) Mitochondria and metabolic enzyme dysfuntion. Abnormalities of the mitochondrial genome or deficiencies of key metabolic enzymes contribute to metabolic impairment and ROS production (Davis *et al.*, 1997; Blass *et al.*, 1990). Metabolic changes are linked to production of ROS and to changes in cellular calcium regulation. Production of ROS is a normal part of the electron transport

chain, and impairment of electron transport (for instance due to inhibition of cytochrome oxidase) promotes ROS production.

4) Abeta itself. An increased number of studies support a central role of Abeta as a molecular link between oxidative stress and AD associated neuronal death (Butterfield, 1997; Varadarajan *et al.*, 2000), and this will be discussed below.

In fact, almost all factors that contribute to oxidative stress are indirectly or directly related to Abeta production or deposition. Hence, it is evident that the genetic mutations mentioned above and other mechanisms (ApoE genotype and redox metal ions) which result in increased Abeta deposition, can contribute to Abeta induced oxidative stress and neurotoxicity (Lauderback *et al.*, 2002; Butterfield *et al*., 2001).

As already mentioned, Abeta can trigger the inflammatory response induced by astrocytes and activated microglia that may be critical for the neurodegenerative process in AD (Figure 4). Rat neuronal-glial cultures exposed to Abeta exhibit increased levels of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) release (Hu *et al.*, 1998; Haas *et al.*, 2002). The latter interacts with superoxide anion forming other free radical, peroxynitrite, which, beside other effects, attacks lipids producing lipid peroxidation (that essentially affects the stability of the membranes). Therefore peroxynitrite can cause oxidative damage of synaptic terminals (Butterfield *et al*., 2002).

Similarly, Abeta peptides can enhance metal-catalyzed oxidation reactions (Dikalov *et al.*, 1999; Yatin *et al.*, 1999), insert into neuronal membranes and initiate free radical damage resulting in protein oxidation, lipid peroxidation, ROS formation, and cellular dysfunction. These processes also produce calcium ion accumulation and subsequent neuronal death (Varadarajan *et al.*, 2000; Butterfield *et al.*, 2001) (Figure 4). Abnormalities in cellular calcium storage affect the ability of cells to handle oxidative stress and to respond to metabolic impairment, a characteristic associated with AD (reviewed in Gibson, 2002).

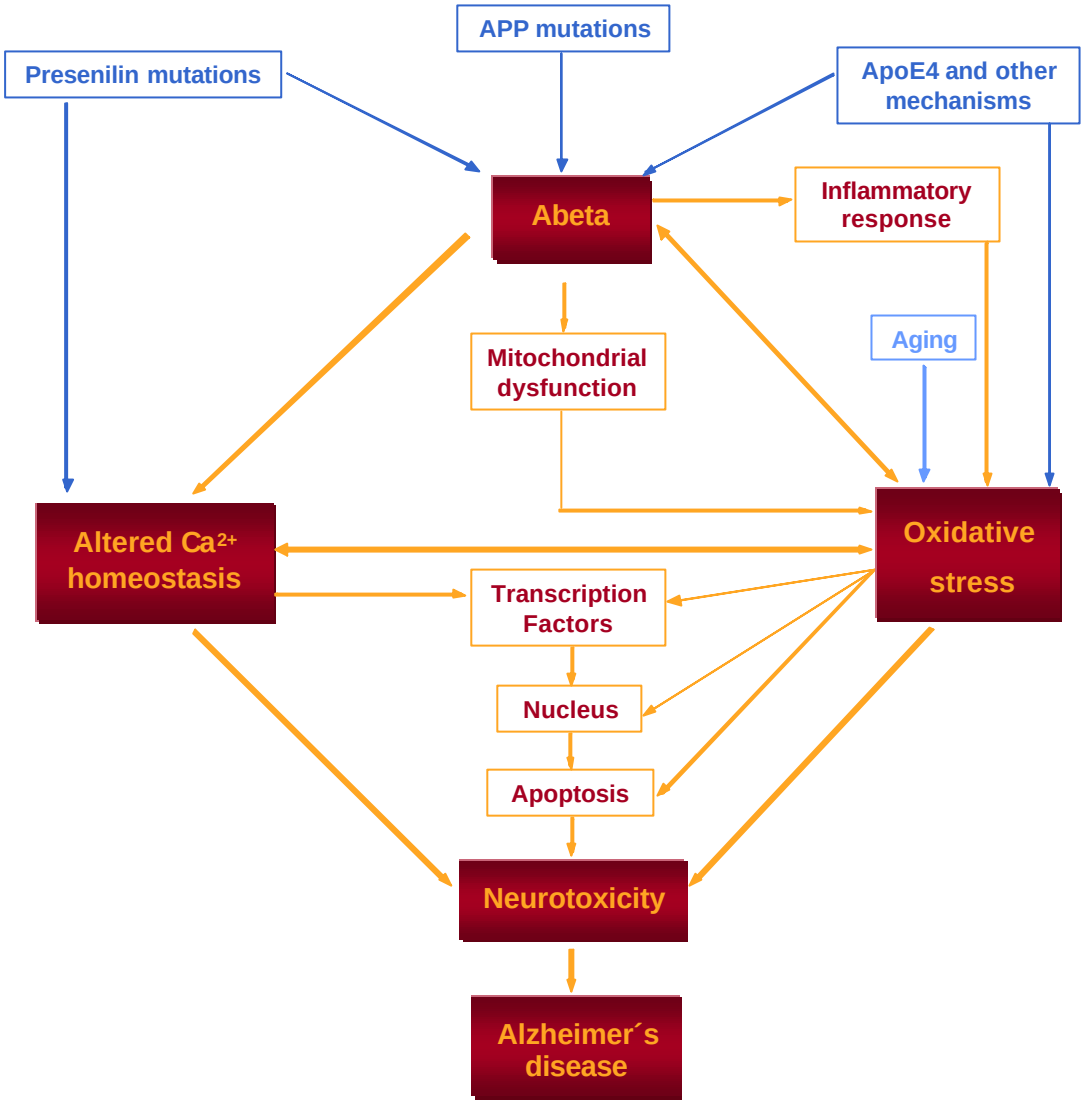

Figure 4. Neurodegenerative process in Alzheimer´s disease. Central role of Abeta in oxidative stress and neurotoxicity (adapted from Butterfield *et al.*, 2001).

Moreover, Abeta leads to oxidative modification and inhibition of several neuronal and glial transmembranes transport systems, such as ion-motive ATPases, glutamate receptors, glucose transporters, GTP-coupled transmembrane signalling proteins and polyamine transporters (Mattson, 1999; Lauderback *et al.*, 2001; Yatin *et al.*, 2001). Loss of each transport systems has deleterious consequences for the neurons, including loss of cellular membrane potential, accumulation of excitotoxic glutamate, decreased glucose availability, decreased intracellular communication and increased neurotoxicity.

Furthermore, several studies have implicated mitochondrial defects in the pathogenesis of AD (Gabuzda *et al*., 1994; Prasad *et al*., 2002). Defective mitochondria lead to reduced production of ATP which results in decreased energy metabolism. For example, decreased glucose uptake coupled with reduced activity of cytochrome oxidase (complex IV) results in increased production of ROS by impaired mitochondria (Mutisya *et al.*, 1994).

As already mentioned, Abeta itself can directly induce the formation of free radicals (Varadarajan *et al.*, 2000, Varadarajan *et al.*, 2001) but it can also increase levels of ROS in cells, such as H_2O_2 (Behl *et al.*, 1994; Huang *et al.*, 1999). These increased levels of H_2O_2 interfere with glucose metabolism, by inhibiting the activity of metabolic enzymes involved in this process (such as pyruvate dehydrogenase, ?-ketoglutarate dehydrogenase enzyme complex and transketoloase) (Chinopoulos *et al.*, 1999; Gibson *et al.*, 2000; Xu *et al.*; 2001, Gibson, 2002). A general reduction in glucose uptake also increases ROS production.

Although, there is evidence that Abeta can contribute to the increased production of oxidative stress in AD, it is noteworthy that oxidative stress and metabolic compromise can lead to altered APP processing resulting in Abeta formation. In fact, oxidative stress induced by H_2O_2 increases Abeta in mammalian lens (Frederikse *et al.*, 1996) and SHSY5Y cells (Misonou *et al.*, 2000; Olivieri *et al.*, 2001). Several reports have analyzed the effect of the metabolic impairment due to inhibition of cytochrome c oxidase (which results in superoxide anion formation) by sodium azide on APP processing. Gasparini *et al.* (1997) showed that sodium azide decreased APPs production from COS-1 cells, and Gabuzda *et al.* (1994) showed that it resulted in the production of a 11.5 KDa C-terminal fragment,

potentially containing the full length Abeta sequence, using COS 1 cells transfected with APP_{695}. In both studies increased levels of the toxic Abeta peptide was implicated. Thus, these results can provide a potential framework to link the reported cytochrome c oxidase deficits in AD (Gibson *et al.*, 1996; Kish *et al.*, 1992; Parker *et al.*, 1994) and the accumulation of Abeta peptides (Vickers *et al.*, 2000).

Taken together, this data suggests a strong correlation between Abeta and oxidative stress. A summary of the neurodegenerative processes in AD brain due to Abeta associated oxidative stress is represented in Figure 4.

1.4 Altered cellular responses: relevance in Alzheimer´s Disease

Cellular stress due to oxidative damage or other stress factors, can promote two fundamental cellular responses: apoptosis (a precisely regulated form of cell death) and heat shock protein induction (which functions to protect cells and to mediate an accelerated recovery following damage). The balance between these two opposing pathways determines whether the cell lives or dies.

1.4.1 Evidence for apoptosis in Alzheimer´s Disease

Several studies suggest that neuronal death in AD is mediated by unregulated apoptosis. Apoptosis, or programmed cell death (PCD), is a highly conserved energy–requiring program for non-inflammatory cell death that is important in both normal physiology and disease. This extremely well organized process involves DNA fragmentation, membrane blebbing, cell shrinkage and disassembly into membrane-enclosed vesicles. These are eliminated by phagocytosis, therefore preventing an inflammatory response to the intracellular components (Grütter, 2000).

Neuropathological studies have shown higher fragmentation of nuclear DNA in neurons, oligodendrocytes, astrocytes and microglia in brains of patients with AD than in age-matched controls (Su *et al.*, 1994; Dragunow *et al.*, 1995; Troncoso *et al.*, 1996). These studies have been interpreted as evidence of neuronal loss in AD due to apoptosis, and that this may be initiated by the toxic Abeta peptide or other inducers of oxidative stress.

Oxidative stress, induced by Abeta or other factors, can trigger apoptosis associated events, activating caspases or increasing expression of apoptotic related proteins. Indeed, ROS induced damage present in AD brains has a dramatic effect on DNA (Lyras *et al.*, 1997) and RNA (Nunomura *et al.*, 2001). Several enzymes involved in DNA repair are dysregulated under oxidative stress conditions affecting the equilibrium of the DNA repair process which can lead to apoptosis.

The poly (ADP-ribose) polymerase (PARP ? a 113-KDa protein) is a DNA repair protein that is activated in response to DNA damage induced by oxidative stress. Single-stranded and double-stranded DNA breaks activate PARP which catalyzes the poly(ADP-ribosyl)ation of this damaged DNA using NAD^+. Nevertheless, overactivation of PARP can result in cell death by necrosis, with a severe ATP depletion and massive consumption of the cofactor NAD^+.

Under oxidative stress conditions PARP is proteolytically cleaved by caspase-3 (an executor of apoptosis) yielding 89- and 24-KDa fragments (Tewari *et al*., 1995). Such cleavage essentially inactivates the enzyme by destroying its ability to respond to DNA strand breaks, and prevents depletion of NAD and ATP, which are thought to be required for latter events in apoptosis. Indeed, PARP cleavage prevents induction of necrosis during apoptosis and ensures appropriate execution of PCD mediated by caspases (Herceg and Wang, 1999). PARP cleavage by caspases is a hallmark of apoptosis.

Besides oxidative stress, Abeta itself appears to also lead to apoptosis (Mattson *et al*., 1998; Carter and Lippa, 2001; Kienlen-Campard *et al*., 2002). Indeed it is believed that the synapse loss and neuronal cell death characteristic of AD (and apoptotic processes) result largely from the neurotoxic effects of Abeta production. Moreover, treatment of rat cortical neurons showed that Abeta 25-35 (the neurotoxic fragment of Abeta peptide) induces increased caspase-3 activity and cleavage of PARP. In the presence of specific caspase inhibitors, Abeta induced PARP cleavage was prevented, but cell death still occurred. This suggests that activation of caspase-3 and other caspase(s) leads to different mechanisms of Abeta induced apoptosis (Harada and Sugimoto, 1999).
In fact, experiments performed with Abeta 25-35 also showed that this active fragment of Abeta, through NO and probably other free radicals, induces

activation of PARP (Harada and Sugimoto, 1999; Strosznadjer *et al.*, 2000). Hence, it is not surprising that Kienlen-Campard *et al.* (2002) demonstrated that neurons undergo apoptosis as soon as they accumulate intracellular Abeta following long term expression of human APP. Concordantly, it has been demonstrated that Abeta 1-42 is toxic to human neurons through activation of p53 and Bax proapoptotic pathway (Aurelio *et al.*, 2000; Zhang *et al.*, 2002).

Furthermore, it has been shown that APP processing and Abeta formation were enhanced during neuronal apoptosis (LeBlanc, 1995; Galli *et al.*, 1998). Also, the finding that the preselinins and APP proteins are substrates for different caspases enhances the possibility that dysregulation of PCD could be involved in the pathogenesis of the disease (Kim *et al*., 1997; Loetscher *et al*., 1997; Pellegrini *et al.*, 1999; Leutz *et al.*, 2002).

Although apoptosis is a fast event and AD is a slow neurodegenerative process, the presented data suggest a link between apoptotic pathways and neurodegeneration in AD. The mechanisms causing cell death may be overlapping with integrated events among the components interacting and contributing to a final pathway for neuron death in the disorder. Apoptosis may not be the leading cause of AD but it can occur as a consequence of it.

1.4.2. Heat shock proteins in Alzheimer´s Disease neuropathology

Heat shock proteins (HSPs) act as molecular chaperones, which are ubiquitous, highly conserved proteins that play pivotal roles in living organisms. At the molecular level, chaperones protect against aggregation, solubilize protein aggregates, assist in the proper folding/refolding of various cellular proteins and target ultimately damaged proteins to degradation (SOti and Csermely, 2000). Chaperones are vital for our cells during their whole lifetime. However, they are needed even more after stress stimuli that induce protein damage. Stress leads to the expression of most chaperones, which therefore are called heat shock or stress proteins. These proteins are induced in living cells, playing a protective role and promoting their recovery following damage. In early stages of AD, a cerebral decrease in energy metabolism, along with a loss of functionally important amino acids, dysregulation of calcium homeostasis and increasing oxidative stress are

assumed to be stress related abnormalities capable of inducing heat shock proteins.

HSPs have been reported to regulate the apoptotic pathway and also play a critical role in protein conformational diseases, such as AD (Carrell and Lomas, 1997; Soto, 1999). In AD a change into beta-sheet conformation of Abeta peptide leads to fibrils assembly and a concomitant toxic effect towards neurons *in vitro* (Lorenzo and Yankner, 1994). HSP70, small HSPs (HSP27 and alpha crystalline B) and HSP40 have been reported to diminish amyloid fibril formation and subsequent toxicity (Shinohara *et al*., 1993; Renkawek *et al*., 1994; Muchowski *et al*., 2000).

Many studies showed that several chaperone proteins, such as HSP70 are aberrantly expressed in AD brains (Yoo *et al.*, 2001). Similarly, there is evidence for increased HSP70 expression in brains from patients with Down´s Syndrome (Yoo *et al.*, 1999). Moreover, expression of these proteins appears to be related to overexpression of APP. In fact, the APP gene promoter contains a heat shock element and altered APP expression, induced by heat shock leads to increased accumulation of Abeta (Johnson *et al.*, 1993). Accordingly Abe *et al*. (1991), using cultured lymphoblastoid cells suggested that APP mRNA was induced by heat shock treatment after the induction of HSP70 mRNA. HSP70-induced APP mRNA suggests a role of heat shock response in the induction of APP expression (Yoo *et al*., 2001). Furthermore, recent studies confirmed that HSP70, HSP90 and HSP32 play a neuroprotective role facilitating Abeta phagocytosis and clearance and cytokine production by activation of microglia (Kakimura *et al.*, 2002).

HSP70, HSP90 and HSP27 can regulate the activities and expression, or both, of apoptotic signalling molecules (Beere, 2001). Indeed, down-regulation or disruption of HSP70 expression results in apoptosis. Mosser *et al.* (1997) demonstrated that this chaperone is able to block apoptosis by inhibiting the apoptotic stress-activated protein kinase SAPK/JNK (c-jun N-terminal kinase) pathway. Clearly the HSP70 family has a crictical contribution to neuronal cell death and hence a strong association with AD.

1.5 Diagnosis for Alzheimer´s Disease

Alzheimer´s Disease is the most common dementia among the elderly, appearing as a new "epidemic" threat to human civilization in the next century. As lifetime expectancy is increasing, the incidence of AD is expected to rise in the near future. Social and economic consequences of this disease will reach immense dimensions. To date, there are no available biological markers and an AD diagnostic is presently based on cognitive testing. There is no assay of objective *ante mortem* biochemical criteria to reveal the pathophysiology of this disease. Although other diagnostic tests such as routine blood examinations, brain imaging techniques, and neuropsychological tests, are used to exclude other disorders. Confirmation of AD is only obtained using *post-mortem* brain tissue. Hence, the development of a test, which is non-invasive, simple, cheap, using easily accessible body fluids, is urgently needed for AD diagnosis. Of note is that since AD is a multifactorial disorder, the use of a single marker is unlikely to be of value. Therefore the use of a combination of markers allows for a more accurate diagnosis.

2. OBJECTIVES

Even though it is not clear how neurons die and synapses are lost in AD, present evidence indicates that APP processing, oxidative stress and Abeta toxicity are the most important mechanisms to explain the development of AD.

This work aimed to adress the effect of oxidative stress on APP processing. Further, given that APP appears to belong to the RIP proteins, phosphorylation dependent events take on added significance. Thus, the first specific objectives were as follows:

1. To study the effect of oxidative stress on APP processing
2. Monitor phorbol ester induction of APP processing under oxidative stress conditions

Although abnormal alterations in protein expression levels (such as HSP in AD brains) have been well document, the link to pathology is elusive. As such, there is a need to develop an experimental model system to study potentially relevant molecular relationships, to correlate molecular markers to AD and this may contribute to the development of novel diagnostic tools. Therefore, and after having established that oxidative stress did in fact play a significant role on APP processing, we went to analyze how oxidative stress and other cellular stresses affected protein expression levels. Our focus was on the long term effect of these exposures, being that AD is a progressive neurodegenerative disorder. The long term goal of this work addresses the monotorization of molecular markers as a useful tool for the early diagnosis of AD. Hence the specific objectives for this section were:

3. To characterize altered protein expression in response to stress agents

3. MATERIALS & METHODS

A list of all reagents and the equipment used is presented in Chapter 7 (ANNEXES). All reagents were cell culture grade or ultrapure.

3.1 Cell culture

Two cell lines were used: non–neuronal COS-1 cells and neuronal-like PC12 cells. COS-1 cells were grown in DMEM supplemented with 10% heat inactivated foetal bovine serum, 1% antimycotic-antibiotic solution and 3.7 g/l $NaHCO_3$. PC12 cells were cultured in RPMI medium supplemented with 10% heat inactivated horse serum, 5% heat inactivated fetal bovine serum, 1% antimycotic-antibiotic mix and 0.85 g/l $NaHCO_3$. Cultures were grown and routinely passaged into tissue culture dishes. Cells were observed with an inverted optical microscope. Before replating, COS-1 cells were dissociated with trypsin. Cell cultures were maintained at 37ºC and 5% CO_2. For experimental procedures COS-1 and PC12 cells were plated on 6-well plates after conting with a hemacytometer. PC12 cells were plated onto poly-L-ornithine (100 ?g/ml). Multiwells were pre-treated for 5 min with poly-L-ornithine, and then washed twice with dH_20 prior to use.

3.2 Experimental models

3.2.1 Oxidative stress model to study APP processing

Sodium azide was used to induce oxidative stress by inhibiting cytochrome c oxidase activity. Experiments were performed in the presence of 2DG which substitutes glucose in the incubation medium and is not metabolized by the cells.

Confluent monolayers of cells were plated as described on section 3.1 and then washed twice with PBS before undergoing different treatments. Cells were treated with various concentrations (0.01-10 mM) of sodium azide (NaN_3) in the presence or absence of 2DG in serum-free, glucose-free and pyruvate-free medium for 2 hr at 37ºC. Cell viability was assessed following treatments with sodium azide by the MTT reduction assay (see section 3.3).

3.2.2 Stimulation of APP processing with phorbol esters

PC12 and COS1 cells of approximately 90% confluence were used. For the phosphorylation dependent response, experiments were performed by co-incubating 50 mM 2-deoxyglucose (2DG), 1 or 10 mM sodium azide (NaN_3) and 0,5 ?M phorbol 12-myristate 13-acetate (PMA), which is a PKC activator, or 1 ?M OA (phosphatase inhibitor), for the same period of time in medium free of serum, glucose and pyruvate.

3.2.3 Stress conditions tested for alterations in protein expression

3.2.3.1 Exposure to sodium azide

Having established that oxidative stress did in fact play a significant role on APP processing, we went to study the long term effect of sodium azide on protein expression. These experiments were carried out by incubating the cells at approximately 80% confluence, with 10 mM sodium azide in serum-free medium for 2hr and allowed to recover during an 18 hr and a 24 hr period.

3.2.3.2 Heat shock

Heat shock experiments were used to induce HSPs (like HSP70). Cells 80% confluents were heat shocked during one and a half hours at 44ºC and allowed to recover in serum-free medium for 18 and 24 hrs.

3.2.3.3 Exposure to Abeta

Abeta 1-40 and Abeta 25-35 were prepared as 1 mM stock solutions in dH_2O. 80% confluent PC12 and COS1 cells were exposed to 10 and 20 ?M Abeta 1-40 or Abeta 25-35 in serum-free medium during 24 hr.

3.3 Measurement of cell viability

Cell viability was determined using the MTT assay. This method is based on reduction by mitochondria of MTT (a water soluble tetrazolium salt) to formazan, an insoluble intracellular purple product. The extent of reduction of MTT was measured spectrophotometrically at 570 nm, according to Mossman (1983).

Briefly, after treatment of the cells, the medium was removed and MTT solution (0.5 mg/ml of serum-free DMEM) was added and incubated for 3hr at 37ºC. Resulting insoluble formazan precipitates were solubilized with acidic isopropanol. Absorbance of converted dye was measured at 570 nm. The viability of the cells was expressed as a percentage of control cells.

3.4 Sample collection and immunodetection

After the appropriate treatments, media and cells were collected into 1% SDS and boiled during 10 min. Cell lysates were sonicated during 30 seconds and samples stored at -20C. Protein determination was performed using a BCA assay (see section 3.4.1) and normalized protein samples were electrophoretically separated by SDS-PAGE (see section 3.4.2). Separated proteins were transferred onto a nitrocellulose membrane (see section 3.4.3) followed by immunoblotting for the specific proteins (see section 3.4.3.2). Detection was carried out using a chemiluminescent method (see section 3.4.3.2.1) or a colorimetric method (see section 3.4.3.2.2). The resulting bands were quantified by densitometry (see section 3.5).

3.4.1 Protein content determination

For protein quantification of cells lysates the BCA assay, based on the use bicinchonic acid (BCA) for the colorimetric detection and quantification of total protein (Smith *et al.*, 1985), was used. This test is based on the capability of proteins to reduce Cu^{2+} to Cu^{+} in an alkaline environment. BCA produces a purple colour in the presence of the reduced Cu^{+} ion. The soluble complexes exhibit a strong

absorbance that can be read at 562nm. The quantitative assays were performed according to the manufacturers instructions and bovine serum albumin (BSA) was used as standard.

Briefly, the quantitative analyses were carried out using 50 ?l of the collected cell lysates (see section 3.4). Known concentrations of the BSA were used as standards and final volumes of 50 ?l were adjusted with 10% SDS and water. Both samples and standards were incubated with 1 ml of working reagent, which is prepared with 50 parts of reagent A to 1 part of the reagent B. All samples were incubated at 37ºC during 30 minutes and then read at 562 nm.

3.4.2 SDS-Polyacrylamide gel electrophoresis

SDS – Polyacrylamide gel electrophoresis (SDS-PAGE) is an analytical technique of electrophoresis of proteins on polyacrylamide gels under conditions that ensure dissociation and characterization of proteins and peptides in mixtures. The anionic detergent SDS is used in combination with a reducing agent (mercaptoethanol) and heat to dissociate proteins before they are loaded on the gel. SDS-PAGE was carried out using a discontinuous buffer system, described by Laemmli (1970), in which a non-restrictive large-pore gel called a stacking gel is layered on top of a resolving gel. In this system each gel layer is of different pH and ionic strength, and the buffer in the reservoirs (running buffer) is different from the gel buffers.

SDS-PAGE was performed using a Hoefer electrophoresis system. The optimum acrylamide concentration of the resolving gel was selected according to the molecular weight of the proteins being monitored (Table 1). The gel was prepared and allowed to polymerize at room temperature. Subsequently, the 3.5% stacking gel solution was prepared and loaded on top of the resolving gel. A Teflon comb was inserted and the gel was left to polymerize at room temperature. Prior to loading, the samples were boiled in SDS gel loading buffer for 3 minutes to ensure protein denaturation. Commercial standards of known molecular weight proteins were used as markers. Proteins were separated electrophoretically (90 mA) for 3-4hr. The amount of total protein loaded varied depending on the protein to be detected (Table 1).

Table 1. Percentage of acrylamide gels used and the corresponding amount of total protein loaded for each.

Protein	MW (KDa)	Acrylamide gel	? g protein
APP/sAPP	ranging from 130-110	7.5%	45/100
PARP	89	8%	70
HSP70	70	10%	35
Tubulin	50	——	35
PP1	37	12%	35

3.4.3 Western blotting

Western blotting is a technique where electrophoretically separated components are transferred from a gel to a solid support. In this work, proteins were electrophoretic transferred to a nitrocellulose membrane (Protan, Schleicher & Schuell) (Burnette 1981). After transfer the proteins were detected using specific antibodies (section 3.4.3.2).

3.4.3.1 Transfer of proteins from the membrane to a solid support

Briefly, the gel was placed in contact with nitrocellulose filter, which were then sandwiched between Whatman 3MM paper, two porous pads, and two plastic supports. The sandwich was immersed in an electrophoresis tank, equipped with standard platinum electrodes, containing transfer buffer. The nitrocellulose filter was placed toward the anode. An electric current of 200mA was applied for about 15 hrs; during this time, the proteins (charged negatively by SDS) migrate from the gel toward the anode and become attached to the nitrocellulose filter. After the transfer of the proteins, the membrane was removed from the sandwich and allowed to dry at room temperature.

3.4.3.2 Immunological detection of the immobilized proteins

Transferred proteins were probed with specific antibodies. Non-specific binding was reduced by blocking with non-fat dried milk (Jonhson *et al.*, 1984). The procedure used was to wet the membranes in TBS solution for 2-3 minutes, proceeded by incubation of the membranes in the blocking solution during 2 hrs. Subsequently, membranes were incubated with an unlabeled primary antibody direct against the target protein, for 2-4 hrs (depending on the primary antibody used). The primary antibody was removed and the filters washed three times (10 minutes) with TBS-T. The membranes were then incubated with a secondary antibody (for 1h30 min), coupled either with horseradish peroxidase or with alkaline phosphatase (enzyme-labelled antibodies). After further washing, the antigen-antibody-antibody complexes on the nitrocellulose filter were detected by autoradiography or *in situ* enzymatic reactions depending on the protein being analyzed (Table 2).

3.4.3.2.1 Chemiluminescent protein detection

ECL or ECL plus are chemiluminescent detection reagents. The ECL (enhanced chemiluminescence) reaction is based on the oxidation of the cyclic diacylhydrazide luminal (Whitehead *et al.*, 1979) and ECL Plus utilizes a technology based on the enzymatic generation of an acridinium ester, which produces a more sensitive light emission of longer duration than ECL.

After incubation with secondary antibody (horseradish peroxidase labelled), the membranes were washed and then incubated with the working mixture of the chemiluminescent detection reagent. The incubation occurred during 1 min for ECL or 5 min for ECL plus at room temperature in a dark room. The membranes were wrapped in SaranWrap and exposed to autoradiography films (Kodak) in an X-ray film cassette. Films were developed and fixed with appropriate solutions (Kodak).

3.4.3.2.2 Colorimetric protein detection

The substrate NBT/BCIP is converted *in situ* into an intense black purple precipitate at the site of enzyme binding (alkaline phosphatase). The substrate solution is stable in the absence of enzyme. The reaction proceeds at a steady rate, thus allowing accurate control of the development of the reaction.

Basically, after incubating the membranes with secondary antibody conjugated with alkaline phosphatase, the membranes were washed and incubated with substrate solution. The blot was developed at room temperature with agitation until the bands were suitably dark (5-30 minutes). The reaction was stopped with stop solution. The membranes were washed with dH_20 and dried for further analysis.

Table 2. Protein antibodies and detection method used.

Protein	First antibody	Secondary antibody	Detection method
APP	22C11	Peroxidase labelled anti-mouse	ECL
sAPP	22C11	Peroxidase labelled anti-mouse	ECL Plus
PARP	Anti-PARP	Peroxidase labelled anti-rabbit	ECL PLus
PP1	Anti-PP1	Peroxidase labelled anti-rabbit	ECL
HSP70	Anti-HSP70	Monoclonal anti-rabbit alkaline phosphatase conjugate	NBT/BCIP
Tubulin	Anti-beta-tubulin	Anti-mouse alkaline phosphatase conjugate	NBT/BCIP

3.5 Statistical analysis

Quantitative analyses were performed using densitometric scanning using a BioRad system. Data were expressed as means ±SE of triplicate determinations, from at least three independent experiments.

4. RESULTS

4.1 The effect of oxidative stress on APP processing

Experiments were carried out as described in section 3.1, in the presence of sodium azide (3.2.1), and the effect short term incubation on cell viability was evaluated (3.3).

4.1.1 Effect of sodium azide on cell viability

PC12 and COS-1 cells at 90% confluency were treated with increasing concentrations of sodium azide, in the presence of 50 mM 2DG, and cell viability was determinated. The results obtained are shown in Figure 5. For both cell lines tested, sodium azide decreased cell viability, with a more pronounced effect at 10 mM concentration. Under these stress conditions, PC12 cells appear to be more sensitive, being that their viability decreased approximately 50%, whereas COS-1 cells are affected much less (approximately 80% viability).

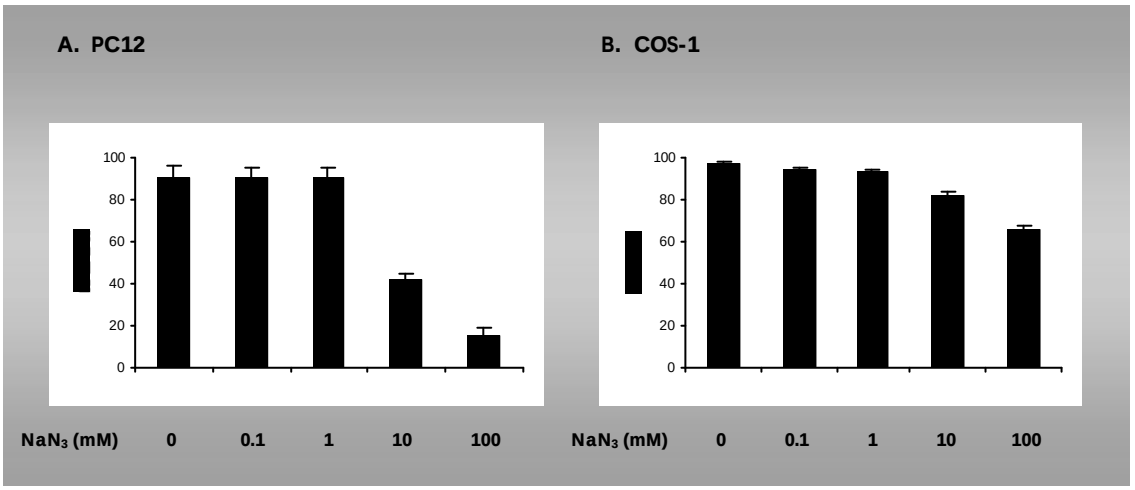

Figure 5. Cell viability was measured using MTT reduction test. Graph A shows the cell viability for PC12 cells and graph B for COS-1 cells.

Given the results obtained, in particular the drastic effect of 100 mM Na N_3 on PC12 cell viability, subsequent experimentation excluded NaN_3 concentrations above 10 mM.

4.1.2 Effect of sodium azide on sAPP production

Oxidative stress was induced using sodium azide, as described above, and cell media and cell lysates were analyzed by immunoblotting (3.4.3) for both cell lines. Figure 6 shows the results of the western blots. Tubulin was used as a control (Figure 6, I) showing no alterations throughout the experiments. Analysis of the medium (Figure 6, III and IV) revealed that for both PC12 and COS-1 cell lines sAPP production decreased upon addition of sodium azide, and overall there was a corresponding decrease in intracellular mature APP (Figure 6, II).

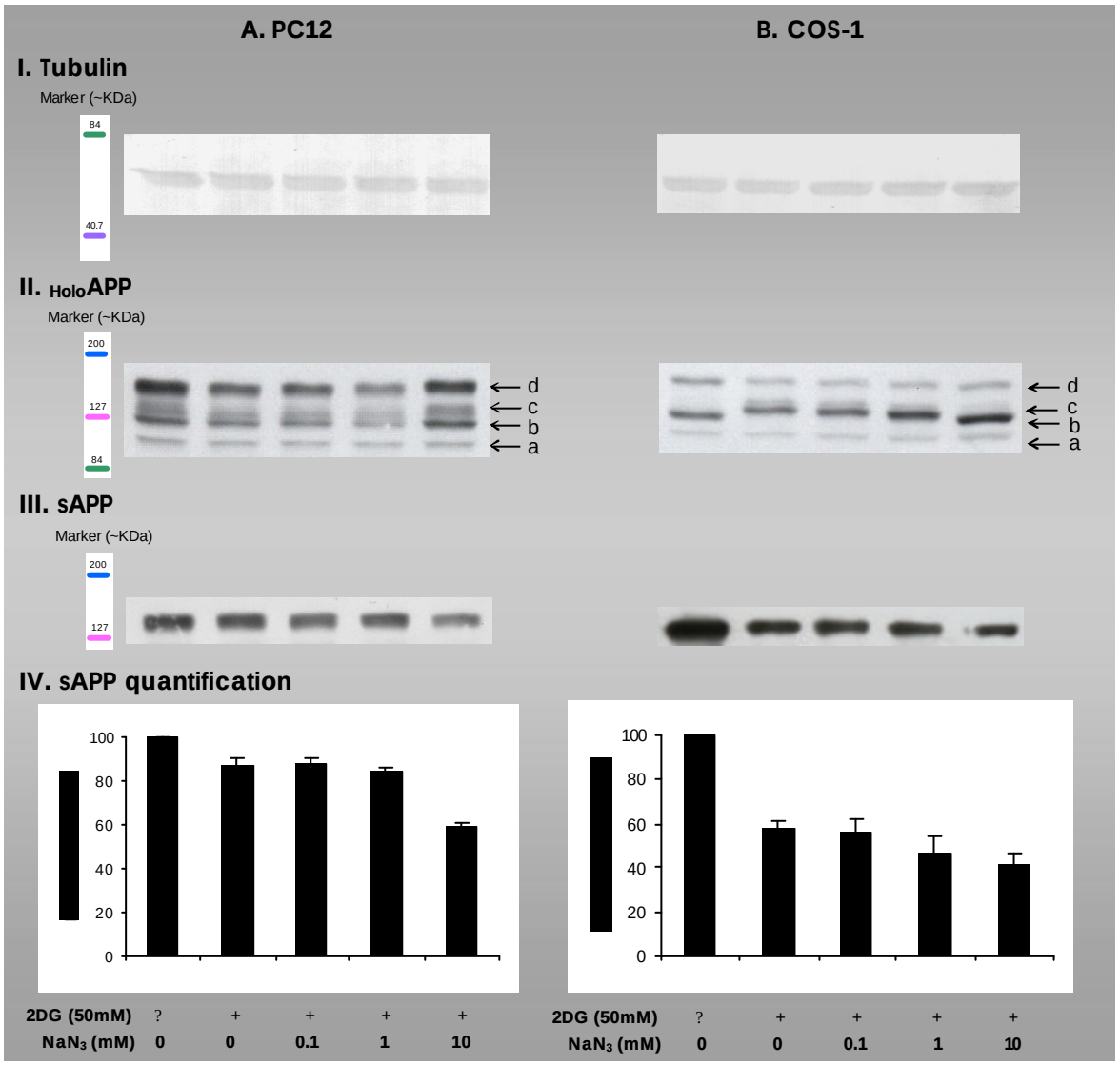

Figure 6. Effect of NaN_3 treatment on PC12 and COS-1 cells. Cells were incubated at 37ºC for 2 hours with different concentrations of NaN_3 in the presence of serum free DMEM and 50 mM 2DG. Cell media and lysates were collected and analyzed by western blot. I. Control blots for beta-tubulin. II Western blots of cellular lysates probed with 22C11 for $_{Holo}$APP. The different bands corresponding to the mature and immature isoforms of APP can be clearly identified: a) immature APP_{695}, b) immature $APP_{751/770}$, c) mature APP_{695} and, d) mature $APP_{751/770}$. III. sAPP secreted into cell medium. IV. Quantification of sAPP secreted (mean ± SEM).

It was noticeable, however that with 10mM NaN_3 the concentration of intracellular APP increased in PC12 cells, possibly due to the lack of processing under toxic conditions. This was particularly observed for the $APP_{751/770}$ isoforms. By contrast the immature APP_{695} isoform appears to remain relatively unaffected. Furthermore, in COS-1 cells the migration pattern of the different APP isoforms is altered immediately upon addition of 2DG, presumably by interfering with APP maturation. This effect was reversed with increasing sodium azide concentrations. Quantification of the results revealed that COS-1 cells appear to be more sensitive to the addition of NaN_3 in relation to sAPP production.

4.2 Phorphol ester stimulation of APP processing under oxidative stress condtions

Having established that sAPP production decreased in both cell lines used, it was tested whether the phosphorylation dependent sAPP increase (section 1.2.1) could still be obtained under stress conditions. Western blot analysis of cell lysates and media of cells exposed to PMA and OA revealed that the rate of sAPP production can be increased 3-4 fold upon addition of PMA and 2 fold upon addition of OA (Figure 7). This is true for COS-1 cells and to a lesser extent PC12 cells. However, upon being subjected to the experimental stress conditions, there is a different response for PMA and OA. In the presence of 1 mM NaN_3 the PMA effect is diminished and the OA effect on sAPP is abolished. However, in the presence of 10 mM NaN_3 both the PMA and OA effects are completely abolished. For COS-1 cells the situation is further complicated, as the mere addition of 2DG appears to abolish the OA induction of sAPP secretion.

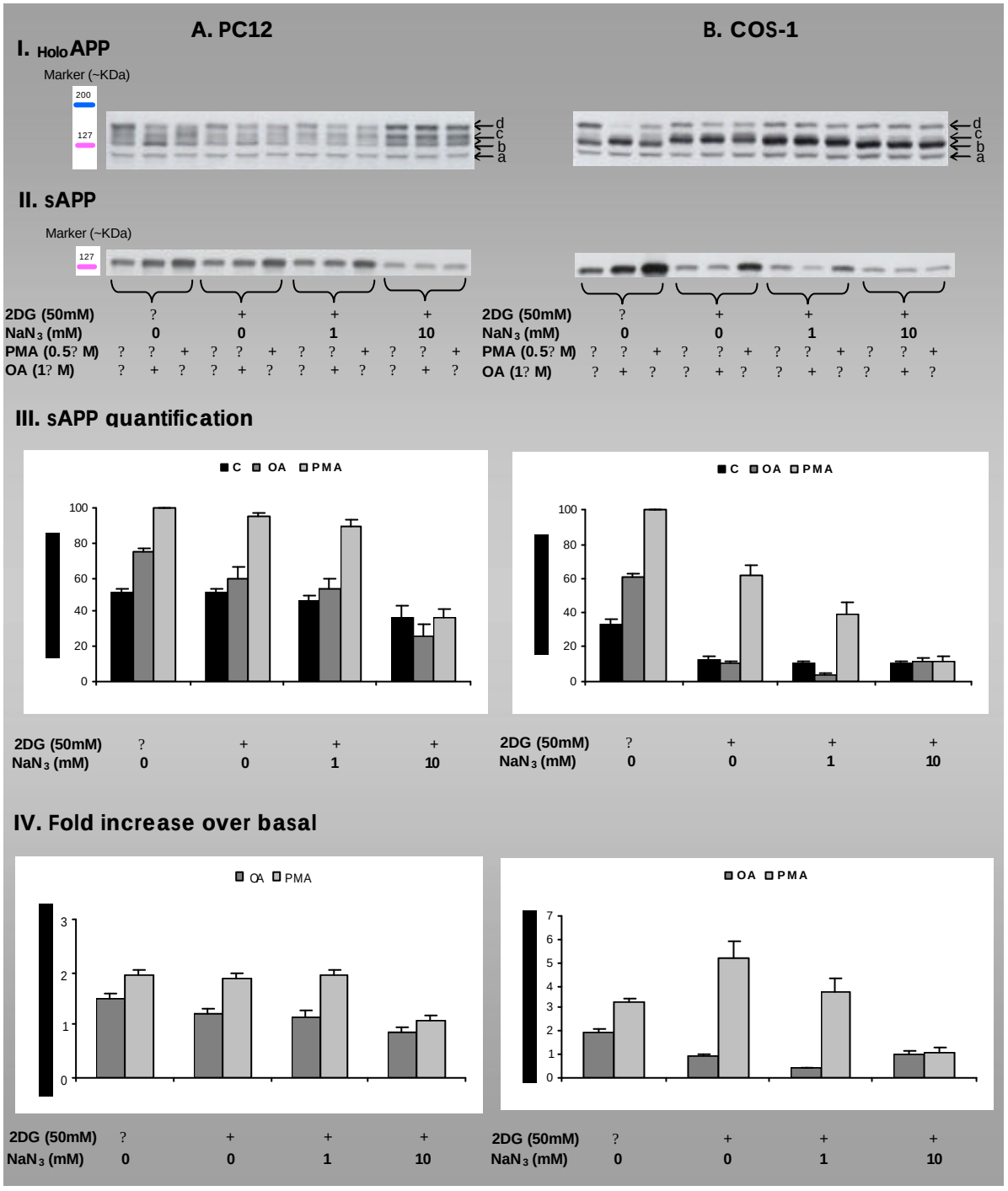

Figure 7. Effect of OA and PMA on PC12 and COS-1 cells treated with NaN_3. Cells were incubated at 37ºC for 2 hours with different concentrations of NaN_3 in serum free DMEM, 50 mM 2DG and 0.5 ?M PMA or 1 ?M OA. Cell media and lysates were collected and analyzed by Western blot. I. $_{Holo}$APP isoforms probed with 22C11. The different bands corresponding to the mature and immature APP isoforms are identified as follows: a) immature APP_{695}, b) immature $APP_{751/770}$, c) mature APP_{695} and, d) mature $APP_{751/770}$. II. sAPP secreted into cell medium. III. Quantification of sAPP secreted. IV. Fold increase over basal.

Analysis of the corresponding cellular lysates (Figure 7) reveals that, as previously shown, increased sAPP production due to PMA or OA addition, causes a decrease in intracellular mature APP. However, at increased NaN_3 concentrations, there is an increase in the intracellular levels of APP, leading us to deduce that APP processing appears to be blocked since it remains unaffected even after the addition of PMA (Figure 7, I). It appears that may be a non-specific toxic effect that is being exerted on the cells under these extreme stress conditions. This also results in diminished sAPP production.

4.3 Alterations in protein expression under cellular stress conditions

Having tested the short term effect of sodium azide on APP processing, we went to study the long term effect on the expression of APP and other proteins. Other cellular stresses relevant to AD were included and their effects on the same proteins were studied. Namely we monitored protein expression levels and induction of apoptosis as detected by PARP cleavage.

4.3.1 Exposure to sodium azide

During the course of these experiments PC12 and COS-1 cells were incubated with 10mM NaN_3 and allowed to recover for 18 and 24 hrs. Methodology used was as outlined in section 3.2.3.1.

The cell lysates were tested for the presence of various proteins by immunoblotting 3.4.3, and also for apoptosis as determined by PARP cleavage products. For both cells lines a decrease in APP expression, in particularly for the 751/770 APP isoforms was detected (Figure 8, I). APP_{695} appears to be less sensitive to exposure to sodium azide stress than the other isoforms. Likewise PP1 expression also decreased in PC12 cells but was almost unaffected in COS-1 cells (Figure 8, II). Similarly HSP70 expression was unaffected in COS-1 cells but decreased in the PC12 cell line. Moreover, apoptosis seems to have occurred as detected by PARP cleavage products (Figure 8, IV). Together, these results indicate that the neuronal-like PC12 cell line appears to be more sensitive to long term exposure to sodium azide than the non-neuronal COS-1 cells.

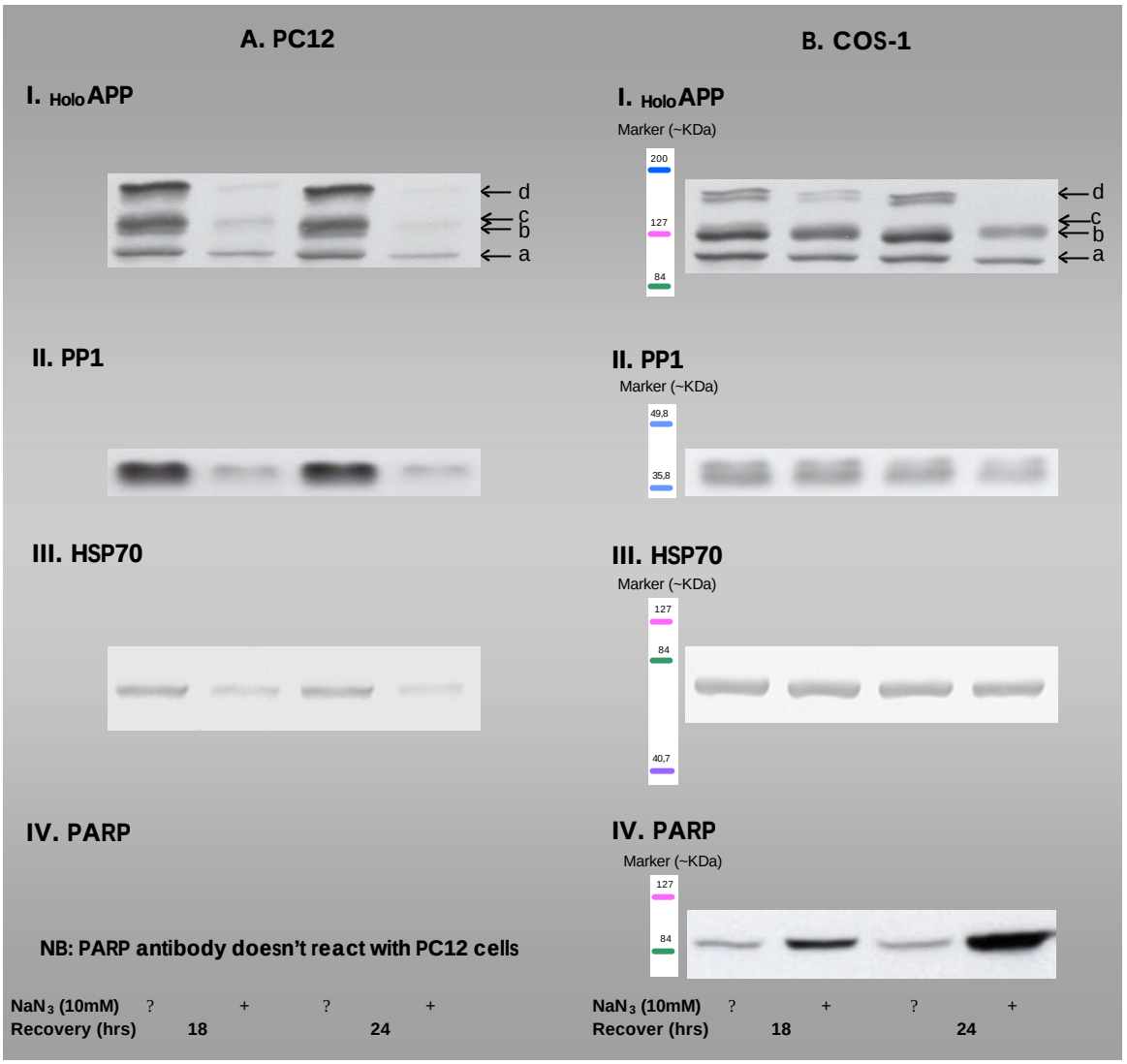

Figure 8. Long term effect of sodium azide on protein expression. PC12 (column A) and COS-1 (column B) cells were treated with 10 mM sodium azide during 2hr in serum-free medium and allowed to recover during 18 and 24 hr. The effect on $_{Holo}$APP isoform expression, PP1, HSP70 and PARP cleavage were also analyzed. The different bands corresponding to the mature and immature APP isoforms are identified as follows: a) immature APP_{695}, b) immature $APP_{751/770}$, c) mature APP_{695} and, d) mature $APP_{751/770}$.

4.3.2 Heat shock

PC12 and COS-1 cells at 80% confluence were heat shocked and allowed to recover during 18 and 24hrs. Procedure was as described on section 3.2.3.2. The results obtained are shown on Figure 9. For both cell lines tested heat shock induced expression of HSP70 as expected (Figure 9, III.). Contrastingly, a decrease in APP expression was observed with heat shock, and it seems that the isoforms 751/770 were again more sensitive than isoform 695 (Figure 9, I.). Also, apparent was a decrease in PP1 expression for both cell lines (Figure 9, II.) and an increase of PARP cleavage under these stress conditions for COS-1 cells (Figure 9, IV.).

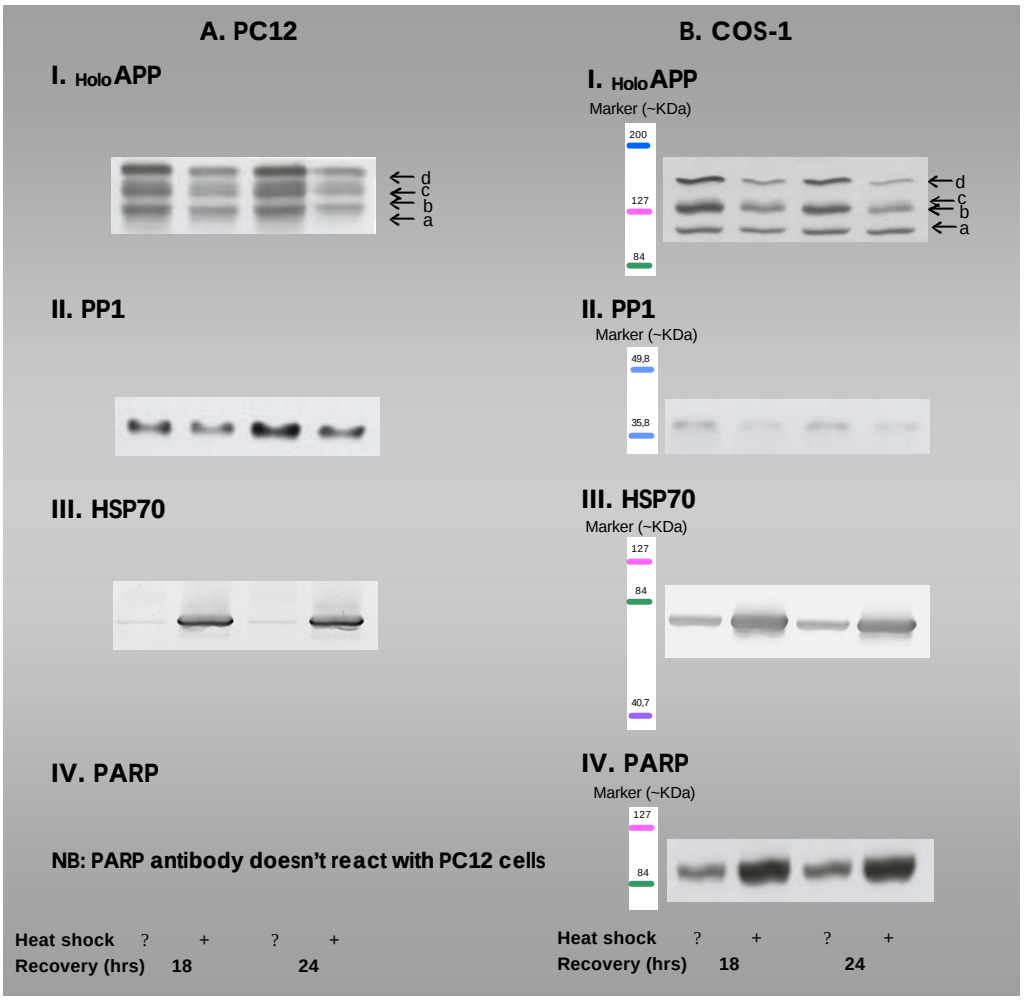

Figure 9. Heat shock effect on protein expression. PC12 (column A) and COS-1 (column B) cells were heat shocked during one hours and a half at 44ºC. Cells were then allowed to recover during 18 and 24 hrs in serum free medium. Cells lysates were collected and analyzed. I. $_{Holo}$APP isoforms probed with 22C11. The different bands corresponding to the mature and immature APP isoforms are identified as follows: a) immature APP_{695}, b) immature $APP_{751/770}$, c) mature APP_{695} and, d) mature $APP_{751/770}$. II. PP1 expression III. HSP70 expression. IV. 85 KDa PARP cleaved fragment.

4.3.3 Exposure to Abeta

PC12 and COS-1 cell were treated with Abeta 25-35 or 1-40 peptides, as mentioned in section 3.2.3.3, and the effects on protein expression were evaluated, (Figures 10 and 11). For both cell lines incubation with Abeta 25-35 leads to increased expression of mature APP_{695} and immature $APP_{751/770}$ isoforms (Figure 10, I), whereas Abeta 1-40 had no effect on APP expression levels at the concentrations tested (Figure 11, I).

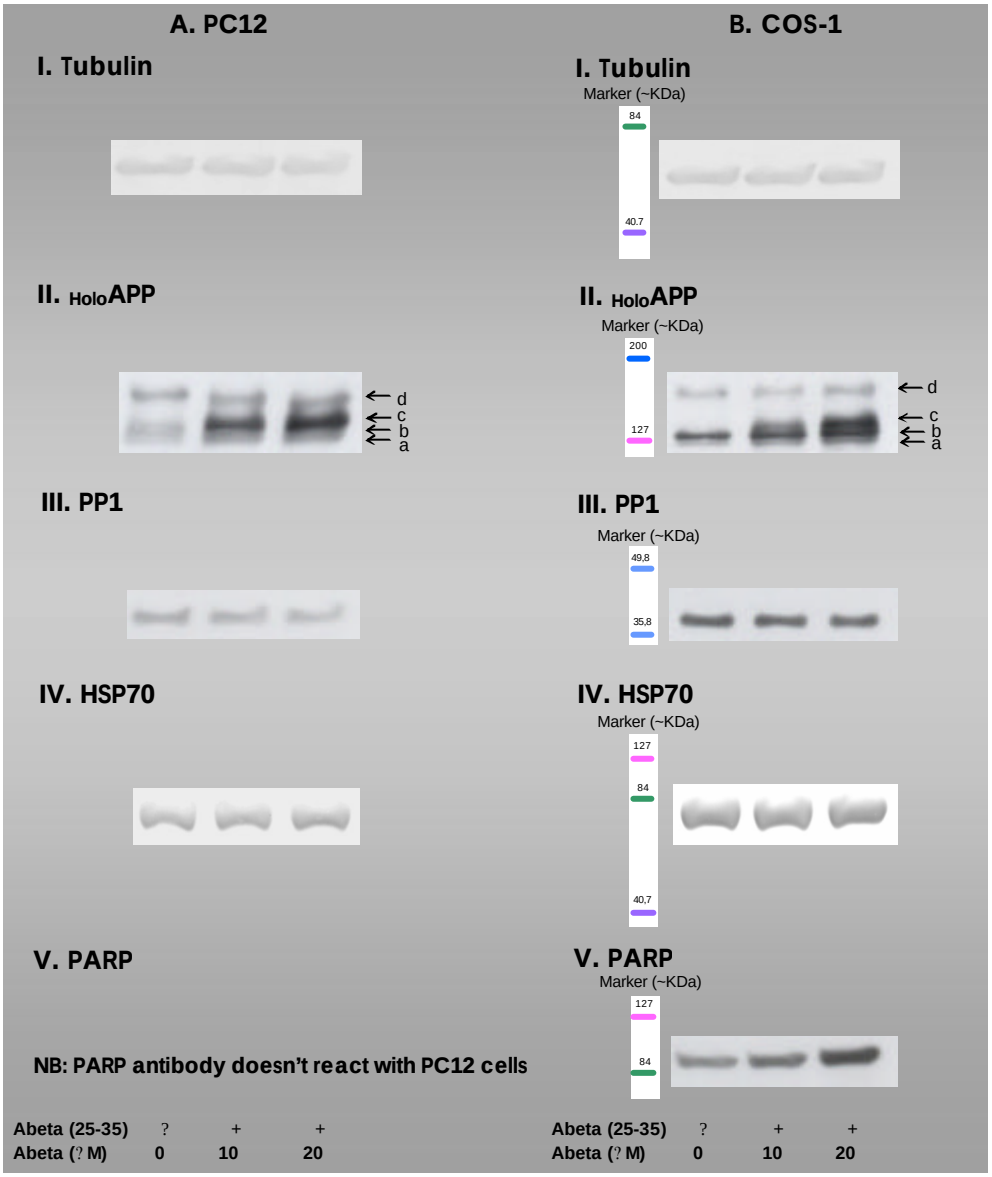

Figure 10. Abeta 25-35 effect on protein expression. PC12 (column A) and COS-1 (column B) cells were treated with 10 ?M and 20 ?M Abeta 25-35 during 24hr in serum free medium. Its effect on Tubulin (I), HoloAPP isoforms (II), PP1 (III), HSP70 (IV) and PARP cleavage (V) were analyzed. The different bands corresponding to the mature and immature APP isoforms are identified as follows: a) immature APP_{695}, b) immature $APP_{751/770}$, c) mature APP_{695} and, d) mature $APP_{751/770}$.

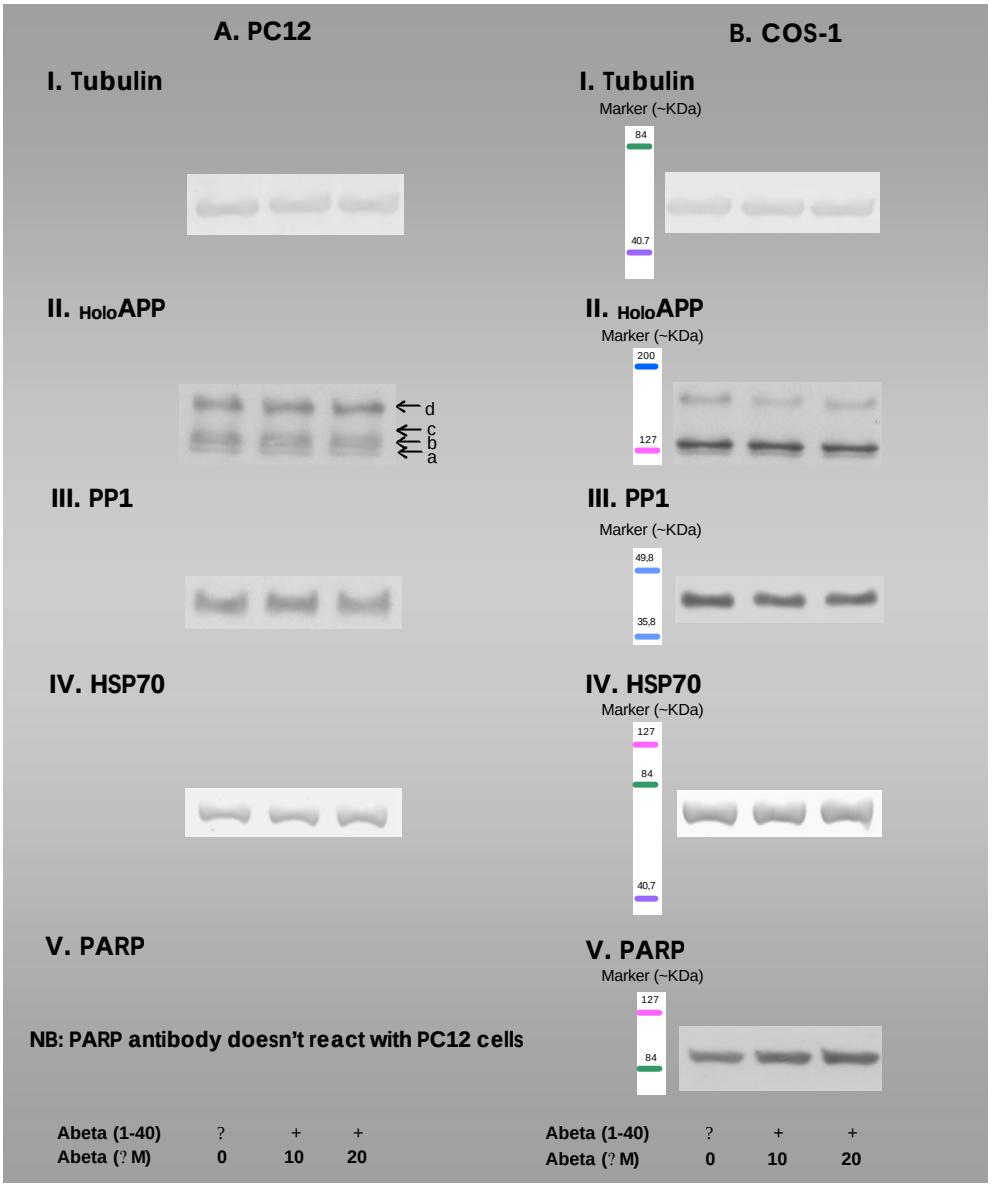

Figure 11. Abeta 1-40 effect on protein expression. PC12 (graph A) and COS-1 (graph B) cells were treated with 10 ?M and 20 ?M Abeta 1-40 during 24hr in serum free medium. Cells lysates were collected and analyzed. I. Control blot to tubulin. II. $_{Holo}$APP isoforms probed with 22C11. The different bands corresponding to the mature and immature APP isoforms are identified as follows: a) immature APP_{695}, b) immature $APP_{751/770}$, c) mature APP_{695} and, d) mature $APP_{751/770}$. PP1?(III), HSP70 (IV) and PARP cleavage (V).

From a molecular point of view other aspects were the same for both cell lines tested. PP1 expression decreased only marginally (Figure 10 and 11). HSP70 expression on the other hand remained unaltered for both cell lines, and this may indicate that these experimental conditions were less stressful for cells than the experiments with sodium azide. The PARP immunoblot revealed increased cleavage with both peptides tested, although less so for Abeta 1-40 (Figure 10, V. and Figure 11, V). Tubulin, used as a control marker showed no alterations under conditions of stress. Overall, under these stress conditions, Abeta 1-40 appears to induce fewer molecular alterations with respect to the markers tested (Figure 11), than Abeta 25-35 (Figure 10).

Section 4 in its entirety reveals that when cell are subjected to stress, molecular alterations, either in protein processing or protein expression can be detected.

5. DISCUSSION & CONCLUSIONS

5.1 Short term effect of sodium azide on the processing of the Alzheimer's amyloid precursor protein

APP is ubiquitously expressed. However, as discussed in the Introduction, Abeta deposition does not occur throughout the body of an AD patient. Additionally, several previous studies point to cell type dependent effects. Hence, throughout the course of this work two cell lines were compared: PC12 and COS-1. PC12 cells are a rat pheochromocytoma-derived line and COS-1 cells are a monkey kidney cell line. In terms of cellular stress various aspects were addressed. First we addressed oxidative stress by adding NaN_3 to the cells in culture, since it is routinely used to mimic oxidative stress (reviewed in Gibson, 2002).

In order to evaluate the overall toxic effect of sodium azide on PC12 and COS-1 cells, cell viability was monitored. Such experiments are important since they indicate whether cells are still under near physiological conditions, or if they are entering cell death due to grossly non-physiological conditions. Cell death is itself an interesting phenomenon given that neuronal degeneration is involved in AD and, if oxidative stress induces cell toxicity and apoptosis, there is circumstantial evidence linking these cellular phenomena to the disease condition.

The cell viability results indicated that PC12 cells are relatively more sensitive to sodium azide than COS-1 cells (Figure 5). In both cases, cell viability only started to be significantly affected at sodium azide concentrations in excess of 10mM. Moreover, and as previously identified by Gasparini *et al*. (1997), oxidative stress, as induced by NaN_3, affects APP metabolism resulting in a decrease in sAPP production in both cells lines (Figure 6, III). However, this decrease is more evident in COS-1. In fact, for COS-1 cells sAPP production falls to less than 50% when compared to the control, whereas for PC12 cells the sAPP production falls to approximately 60% of control. Interestingly, COS-1 cells were more resistant, in terms of cell viability, to the addition of sodium azide. Hence, COS-1 cells are more resistant to the addition of NaN_3 but show greater sensitivity in terms of sAPP production. PC12 cells, on the other hand, are more sensitive to sodium azide addition but do not show such a sharp decrease in sAPP production upon

addition of NaN_3. Hence, both cell lines tested respond differently to sodium azide in terms of cell viability and sAPP production.

Interestingly, the decrease in sAPP secretion into the medium is also accompanied by a decrease in intracellular mature APP. The latter is contrary to what would be expected from a simple APP processing perspective. That is, if sAPP production decreases, one would expect mature APP to accumulate in the cell. Thus, the results imply that, under these experimental conditions, the supply of mature APP is a limiting factor to sAPP secretion, and it would appear that APP maturation is also being affected.

The intracellular levels of APP were observed to fluctuate considerably during the course of these experiments. Under the most extreme stress conditions tested (10 mM sodium azide), intracellular APP appears to be significantly increased (particularly $APP_{751/770}$) and sAPP production is further decrease. Again, this observation runs contrary to the 'a priori' expected correlation. One would expect that a higher concentration of intracellular APP might result in increased processing and accordingly yield increased sAPP production. Therefore, the observed decrease in sAPP production is likely to reflect the oxidative stress induced by sodium azide.

Of particular note in COS-1 cells, a shift in APP isoform mobility on SDS-PAGE could be detected in the presence of 2DG (Figure 6, II). These results suggest that the observed experimental alterations in APP metabolism may not result from mitochondrial dysfunction, as postulated by other authors, but are probably related to effects induced by the use of the 2DG glucose analogue. It probably exerts additional effects, as detected by altered mobility of $_{Holo}$APP. In particular, there is an apparent mobility shift for the immature 751/770 isoforms in COS-1 cells. It appears, therefore, that for this cell line, these APP isoforms are selectively affected under the stress conditions tested. It has previously been postulated (McFarlane *et al*., 1999) that different glycoforms of the protein may exist, resulting in significant heterogeneity of the protein, which in turn has important consequences in its processing and function. This being the case, APP_{695} and $APP_{751/770}$ could potentially be responding differently under similar cellular stress conditions. Whether this subsequently affects the availability of $_{Holo}$APP for processing remains to be elucidated. Nonetheless, identifying altered

glycosylation is potentially very interesting, as altered protein glycosylation has been associated with AD (McFarlane *et al*., 1999). Of further interest is the suggestion that differential glycosylation of cerebrospinal fluid acetylcholinesterase in AD may serve as a potential diagnostic marker for the disease. Furthermore, protein glycosylation state appears to be important in protein sorting and targeting to specific cell sites, such as the cell surface (Nguyen and Amera, 1996). However, it remains unclear how this altered glycosylation arises and whether oxidative stress could be a contributing factor. While changes in mobility for immature $APP_{751/770}$ were apparent, this was not so for mature APP isoforms in COS-1 cells, although the amount of mature $APP_{751/770}$ decreased dramatically (Figure 6, II). The APP isoforms in the PC12 cell line did not exhibit such a mobility shift in the presence of 2DG. Again bringing to the fore potential differences in APP metabolism between the non-neuronal and neuronal like cell lines.

In all these experiments we used sodium azide, which is typically used as an inducer of oxidative stress, being an inhibitor of cytochrome c oxidase. Sodium azide provoked a decrease in sAPP production, and a decrease in intracellular mature APP. However, upon addition of 10mM NaN_3, intracellular APP concentration appears to increase. Increased levels of APP expression have been associated with other stress conditions, such as incubation with Abeta 25-35 (Figure 10, II) or addition of staurosporine (unpublished observations). Interestingly, higher concentrations of NaN_3 in COS-1 cells reverts the effect of 2DG on APP isoform mobility. Taken together these results suggest that sodium azide is not a specific inducer of oxidative stress. It appears that our results also support the observation that sodium azide may be rather non-specific (Hedin and Fowler, 2000). Nonetheless, NaN_3 is clearly inducing cellular stress and affecting APP processing; as such it provides an interesting 'in vitro' model to study APP processing under conditions of stress.

5.2 Dissociation of APP processing pathways under conditions of cellular stress

The effect of phosphorylation on APP metabolism and processing has been well documented and, as already discussed, several lines of evidence have suggested that oxidative stress is involved in Alzheimer Disease. Both altered phosphorylation and oxidative stress affect Abeta production, which is thought to be a key aspect in the pathology of AD. Additionally, given the potential role that APP may play as a signal transduction molecule, with consequences for signal transduction therapeutics (da Cruz e Silva *et al*., 2003), we went on to study the effect of PMA and OA under the stress conditions tested (Figure 7). It is important to understand and characterize cellular responses to phorbol esters under stress conditions, as cells in AD patients are also highly likely to be under stress. Previous studies showing an increase in sAPP production in response to phorbol esters (Buxbaum *et al*., 1993; da Cruz e Silva *et al*., 1993; da Cruz e Silva *et al*., 1995a) implicate a concomitant decrease in Abeta, thus highlighting the potential interest of this observation for therapeutics. Hence, we set out to investigate whether phosphorylation dependent sAPP stimulation could still be detected under conditions of stress. Figure 7 (IV) shows that for PC12 cells the PMA response (2-fold increase in sAPP) still occurs at 1mM but not at 10 mM NaN_3. The case is similar for COS-1 cells, although the stimulation is around 4-fold. The peak in the PMA response for COS-1 cells upon addition of 2DG reflects a lower basal level of sAPP, as described in the previous section. Consistent for both cell lines is the finding that at 10 mM sodium azide the PMA dependent sAPP increase is abolished.

As already discussed, PMA-stimulation of sAPP production is important as it can potentially lead to decreased Abeta formation. Under these experimental conditions, where PMA-induced sAPP production is maintained, there is a strong indication that even under oxidative stress conditions PMA can still potentially reduce Abeta production. That is, the potentially therapeutically beneficial PMA effect is still observed under oxidative stress conditions. The response to OA however, and unexpectedly, is different. Typically, OA (like PMA) produces an increase in sAPP production. This response was abolished, in both cell lines tested,

when they were subjected to sodium azide induced stress (Figure 7, II). Thus, under stress conditions we can dissociate two phosphorylation sensitive events affecting sAPP production. One is sensitive to PMA and OA, and the other is only sensitive to PMA. Alterations in the biochemical responses under stress conditions may offer interesting clues to the molecular basis of AD and therapeutic approaches.

5.3 Long term effect of exposure to sodium azide and heat shock

In the next stage of this project, and after establishing that sodium azide induced stress did in fact produce interesting short term effects on APP processing, we went on to evaluate how sodium azide and other cellular stresses relevant to AD affected the expression of various proteins. We chose to monitor proteins that appear to be relevant from an AD pathology perspective. PARP cleavage was also included in our molecular analysis, since apoptosis has been associated with AD.

Treatment of cells with NaN_3 or heat exposure, followed by a recovery period produced various adverse effects, detectable after 18hr and 24hr. For COS-1 cells both insults appeared to induce apoptosis, as determined by the PARP immunoblot (Figure 8 and 9, IV). Likewise, both treatments produced a decrease in $_{Holo}$APP and PP1 in both cell lines. Of particular note is the finding that the $APP_{751/770}$ isoforms appear to be particularly affected (for example Figure 8 (I) for COS-1 cells, where the disappearance of the above mentioned isoforms is virtually absolute). HSP70 induction did not differ significantly for the two types of stress being considered here. With heat shock a typical heat shock response was observed (Figure 9, III), HSP70 induction is obvious for both cell lines). However, HSP70 does not alter at all in COS-1 cells following NaN_3 exposure and, by contrast, it decreases in PC12 when subjected to NaN_3 (Figure 8, III).

Comparing the long term effect of exposure to sodium azide and heat shock, it would appear that heat shock is more aggressive in terms of altered expression of HSP70 and PP1. In terms of APP expression however, NaN_3 appears to be more detrimental. Lastly, if one compares PC12 and COS-1 cells they appear to be similarly affected under the conditions tested.

5.4 Long term effect of exposure to Abeta

Having established that NaN_3 and heat stress can provoke a molecular response with relevance to AD, we went to test the effect of two pathologically relevant candidates, $Abeta_{25-35}$ and $Abeta_{1-40}$. The particular interest in these peptides is that they can be deposited in senile plaques. Both have been used extensively for this type of experimentation, particularly the latter. The results obtained for PP1, HSP70 and PARP (the latter only in COS-1 cells) revealed only small alterations of the proteins in the cellular lysates. This rather suggests that the cellular stress induced by Abeta is moderate by comparison to the other stresses tested. Of particular significance is the hitherto undescribed result that $Abeta_{25-35}$ considerably increases expression of APP itself. This is important on several levels:

i. APP appears to be similar to RIP proteins. The latter produce peptides that can regulate their own expression. Since Abeta exposure can induce APP expression, this provides indirect evidence for APP as a RIP protein.
ii. Potentially, excess Abeta produced in AD patient brains can induce increased expression of APP and increased Abeta production in a 'positive feedback' pathological mechanism.
iii. Individuals with Down's syndrome, having an extra copy of the APP gene, have early onset AD, exhibiting a direct correlation between dose effect and age of AD onset.
iv. The fact that AD is a progressive disorder, correlates with Abeta inducing APP expression leading to further Abeta production in a time dependent manner.

Hence, it would appear that the pathological consequence of the appearance and deposition of the first Abeta peptides can be considerable. Indeed, a small amount of Abeta can have direct consequences and although Abeta addition appears to be less traumatic to the cell, compared to other stresses tested, it may in fact be more toxic to the cell due to a cumulative effect.

5.5 Potential application of molecular markers

The long-term objective of the studies encompassing these preliminary experiments is to characterize potential molecular markers that can be useful in the diagnosis of the early stages of AD. Since AD is a multifactorial disorder, the use of a single marker is unlikely to be of value. Therefore, the use of a carefully selected combination of markers should allow for more accurate diagnosis. The results described, although not conclusive, can provide clues as to how some proteins are respond to different types of stress linked to the pathogenesis of AD. In a preliminary approach we can say that under extreme stress conditions (sodium azide and heat shock experiments) there was a decrease in APP expression, in particular on the 751/770 isoforms, for both cell lines (Figures 8 and 9, I), accompanied by a substantial increase in PARP cleavage (apoptosis). Apparently, the APP_{695} isoform is less sensitive to stress than the $APP_{751/770}$ isoforms. Hence, APP may constitute itself an interesting molecular diagnostic tool for AD. The other proteins monitored (PP1, HSP70 and PARP) could potentially also turn out to be useful markers. Clearly, the proteins studied do not represent an extensive array of all potential molecular protein markers for AD. Nonetheless, this approach may identify potential molecular markers useful for the early AD diagnosis and other dysfunctions.

5.6 Final conclusions

From the experiments presented here several conclusions can be drawn:

1. Different cell lines respond differently to the same stress, both in terms of protein processing and altered protein expression. Essentially, upon addition of sodium azide PC12 cell viability is more readily affected, whereas in terms of APP processing and increased processing in the presence of phorbol esters, COS-1 cells appear to be more sensitive.
2. Short term treatment of cells with sodium azide appears to be toxic on several levels, namely, APP processing, APP maturation and cell viability.
3. Different APP isoforms respond differently to similar insults. For example, band shift in response to 2DG addition, is more pronounced for the 751/770 isoforms. Again the same isoforms appears to be more sensitive, disappearing upon exposure to e.g. NaN_3.
4. PMA induction of sAPP is maintained under conditions of stress but OA induction is abolished. It would appear therefore that one can dissociate APP processing pathways under conditions of stress. This may have implications for AD patients whose cells are under stress.
5. Exposure to sodium azide and heat shock appear to constitute more aggressive stresses than exposure to Abeta peptides.
6. Addition of Abeta 25-35 causes increased APP expression. This can be of significant pathological consequence since neuronal cells can be exposed to Abeta to varying degrees.
7. Finally, the methodology presented identified molecular markers for cellular stress, potentially useful in AD. Clearly further work is necessary in order to validate these results in a more applied setting.

6. BIBLIOGRAPHY

Abe, K., St. George-Hyslop, P. H., Tanzi, R. E. and Kogure, K. (1991). Induction of amyloid precursor protein mRNA after heat shock in cultured human lymphoblastoid cells. *Neurosci. Lett.* **125**, 169-171.

Alzheimer, A. (1907). Uber eine eigenartige Erkankung der Hirnrinde. *Allg. Zschr. F Psychiatr. Psychisch-Gerichlt. Mediz.* **64**, 146-148.

Aurelio, O. N., Kong, X. T., Gupta, S. and Stanbridge, E. J. (2000). p53 mutants have selective dominant-negative effects on apoptosis but not growth arrest in human cancer cell lines. *Mol. Cell. Biol.* **20**, 770-778.

Beere, H. M. (2001). Stressed to death: regulation of apoptotic signalling pathways by the heat-shock proteins. *SciSTKE.* **2001**(93), RE1.

Behl, C., Davis, J. B., Lesley, R. and Schubert, D. (1994). Hydrogen peroxide mediates amyloid beta protein toxicity. *Cell* **77**, 817-827.

Blass, J. P., Baker, A. C., Ko, L. and Black, R. S. (1990). Induction of Alzheimer antigens by an uncoupler of oxidative phosphorylation. *Arch. Neurol*. **47**, 846-869.

Burnette, W. N. (1981). "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112; 195.

Butterfield, D. A. (1997). Beta-Amyloid-associated free radical oxidative stress and neurotoxicity: implications for Alzheimer´s disease. *Chem. Res. Toxicol*. **10**, 495-506.

Butterfield, D. A., Drake, J., Pocernich, C. and Castegna, A. (2001). Evidence for oxidative damage in Alzheimer´s disease brain: central role for amyloid ?-peptide. *TRENDS Mol. Med.* **7**, 548-554.

Buxbaum, J. D., Gandy, S. E., Cicchetti, P., Ehrlich, M. E., Czernick, A. J., Fracasso, R. P., Ramabhadran, T. V., Unterbeck, A. J. and Greengard, P. (1990). Processing of Alzheimer beta/A4 amyloid precursor protein: modulation by agents that regulate protein phosphorylation. *Proc. Natl. Acad. Sci. USA* **87**, 6003-6006.

Buxbaum, J. D., and Greengard, P. (1996). Regulation of APP processing by intra- and intercellular signals. *Ann. NY Acad. Sci.* **777**, 327-331.

Buxbaum, J. D., Koo, E. H. and Greengard, P. (1993). Protein phosphorylation inhibits production of Alzheimer amyloid beta/A4 peptide. *Proc. Natl. Acad. Sci. USA* **90**, 9195-9198.

Buxbaum, J. D., Liu, K. N., Luo, Y., *et al.* (1998). Evidence that tumor necrosis factor alpha converting enzyme is involved in regulated alpha-secretase cleavage of the Alzheimer amyloid precursor protein. *J. Biol. Chem.* **273,** 27765-27767.

Cai, X.-D., Golde, T. E. and Younkin, S. G. (1993). Release of excess amyloid ? protein from a mutant amyloid ? protein precursor. *Science* **259**, 514-516.

Caporaso, G. L., Gandy, S. E., Buxbaum, J. D., Ramabhadran, T. V. and Greengard, P. (1992). Protein phosphorylation regulates secretion of Alzheimer beta/A4 amyloid precursor protein. *Proc. Natl. Acad. Sci. USA* **89**, 3055-3059.

Caputi, A., Barindelli, S., Pastorino, L., Cimino, M., Buxbaum, J. D., Cattabeni, F. and Di Luca, M. (1997). Increased secretion of the amino-terminal fragment of amyloid precursor protein in brains of rats with a constitutive up-regulation of protein kinase C. *J. Neurochem*. **68**, 2523-2529.

Carrell, R. W. and Lomas, D. A. (1997). Conformational disease. *Lancet* **50**(9071), 134-138.

Carter, J. and Lippa, C. F. (2001). Beta-amyloid, neuronal death and Alzheimer´s disease. *Curr. Mol. Med*. **1**(6), 733-777.

Chartier-Harlin, M. C., Crawford, F., Houlden, H., Warren, A., Hughes, D., Fidani, L., *et al.* (1991). Early-onset Alzheimer's disease caused by mutations at codon 717 of the ?-amyloid.precursor protein gene. *Nature* **353**, 844-846.

Checler, F. (1995). Processing of the ?-amyloid protein and its regulation in Alzheimer´s disease. *J. Neurochem.* **65**, 1431-1444.

Chinopoulos, C., Tretter, L. and Adam-Vizi, V. (1999). Depolarization of in situ mitochondria due to hydrogen peroxide-induced oxidative stress in nerve terminals: inhibition of alpha-ketoglutarate dehydrogenase. *J. Neurochem*. **73**, 220-228.

Citron, M., Oltersdorf, T, Haass, C., McConlogue, L., Hung, A. Y., Seubert, P., Vigo-Pelfrey, C., Lieberburg, I. and Selkoe, D. J. (1992). Mutation of the ?-amyloid precursor protein in familial Alzheimer´s disease increases ?-amyloid production. *Nature* **360**, 672-674.

Colton, C. A. and Gilbert, D. L. (1987). Production of superoxide anions by a CNS macrophage, the microglia. *FEBS Lett.* **223**, 284-288.

da Cruz e Silva, E. F., da Cruz e Silva, O. A. B., Zaia, C. T. V. and Greengard, P. (1995a). Inhibition of protein phosphatase 1 stimulates secretion of Alzheimer amyloid precursor protein. *Mol. Med.* **1,** 535-541**.**

da Cruz e Silva. E. F., Fox. C. A., Ouimet. C. C., Gustafson. E., Watson. S. J. and Greengard, P. (1995b). Differential expression of protein phosphatase 1 isoforms in mammalian brain. *J. Neurosci.* **15**, 3375-3389**.**

da Cruz e Silva, O. A. B., Iverfeldt, K., Oltersdorf, T., Sinha, S., Lieberburg, I., Ramabhadran, T. V., Suzuki, T., Sisodia, S. S., Gandy, S. and Greengard, P. (1993).

Regulated cleavage of Alzheimer beta-amyloid precursor protein in the absence of the cytoplasmic tail. *Neuroscience* **57,** 873-877.

da Cruz e Silva, O. A. B., Fardilha, M., Henriques, A. G., Rebelo, S., Guerra e Paz, S. and Da Cruz e Silva, E. F. (2003). Signal transduction therapeutics: A novel approach in Alzheimer´s disease. J. Mol. Neurosci. (in press).

Davis, R. E., Miller, S., Herrnstadt, C., Ghosh, S. S., Hahy, E., Shinobu, L. A., Galasko D., Thal, L. J., Beal, M. F., Howell, N. and Parker, W. D. Jr. (1997). Mutations in mitochondrial cytochrome c oxidase genes segregate with late-onset Alzheimer disease. *Proc. Natl. Acad. Sci. USA* **94**, 4526-4531.

De Strooper, B., Saftig, P., Craessaerts, K., Vanderstichele, H., Guhde, G., Annaert, W., Von Figura, K. and Van Leuven, F. (1998). Dificiency of preselinin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature* **391**, 387-390.

Dikalov, S. I., Vitek, M. P., Maples, K. R. and Mason, R. P. (1999). Amyloid beta peptides do not form peptide-derived free radicals spontaneously, but can enhance metal-catalyzed oxidation of hydroxylamines to nitroxides. *J. Biol. Chem*. **274**, 9399.

Dodart, J. C., Mathis, C., and Ungerer, A. (2000). The beta-amyloid precursor protein and its derivates: from biology to learning and memory processes. *Rev. Neurosci*. **11**, 75-93.

Dragunow, M., Faull, R. L., Lawlor, P., Beilharz, E. J., Singleton, K., Walker, E. B., et al. (1995). In situ evidence for DNA fragmentation in Huntington´s disease striatum and Alzheimer´s disease temporal lobes. *Neuroreport* **6**, 1053-1057.

Duff, K., Eckman, C., Zehr, C., Yu, X., Prada, C. M., Perez-tur, J., Hutton, M., Buee, L., Harigaya, Y., Yager, D., *et al.* (1996). Increased amyloid-beta42(43) in brains of mice expressing mutant presenilin 1. *Nature* **383**, 710-713.

Ebinu, J. O., and Yankner, B. A. (2002). A RIP tide in neuronal signal transduction. *Neuron* **34**, 499-502.

Edelberg, H. K. and Wei, J. Y. (1996). The biology of Alzheimer´s disease. *Mech. Ageing Dev.* **91**, 95-114.

Esler, W. P. and Wolfe, M. S. (2001). A portrait of Alzheimer secretases-new features and familial faces. *Science* **293**, 1449-1454.

Frederikse, P. H., Garland, D., Zigler, J. S. and Piatigorsky, J. (1996). Oxidative stress increases production of beta-amyloid precursor protein and beta amyloid (Abeta) in mammalian lenses, and Abeta has toxic effects on lens epithelial cells. *J. Biol. Chem*. **271**, 10169-10174.

Gabuzda, D., Busciglio, J., Chen, L. B., Matsudaira, P. and Yankner, B. A. (1994). Inhibition of energy metabolism alters the processing of the amyloid precursor protein and induces a potentially amyloidogenic derivate. *J. Biol. Chem.* **269**, 13623-13628.

Gabuzda, D., Busciglio, J. and Yanker, B. A. (1993). Inhibition of beta-amyloid production by activation of protein kinase C. *J. Neurochem.* **61**, 2326-2329.

Galli, C., Piccini, A., Ciotti, M. T., Castellani, L., Calissano, P., Zaccheod, D. and Tabaton, M. (1998). Increased amyloidogenic secretion in cerebrall granule cells undergoing apoptosis. *Proc. Natl. Acad. Sci. USA* **85**, 1247-1252.

Gandy, S. E., Czernik, A. J. and Greengard, P. (1988). Phosphorylation of Alzheimer disease amyloid precursor peptide by protein kinase C and Ca2+/calmodulin-dependent protein kinase II. *Proc. Natl. Acad. Sci. USA* **85**, 6218-6221.

Gasparini, L., Racchi, M., Benussi, L., Curti, D., Binetti, G., Bianchetti, A., Trabucchi, M. and Govoni, S. (1997). Effect of energy shortage and oxidative stress on amyloid precursor protein metabolism in COS cells. *Neurosci. Lett*. **231**(2), 113-117.

Genoux, D., Haditsch, V., Knobloch, M., Michalon, A., Storm, D. and Mansuy, I. M. (2002). Protein phosphatase 1 is a molecular constraint on learning and memory. *Nature* **418**, 970-975.

Gibson, G. E. (2002). Interactions of oxidative stress with cellular calcium dynamics and glucose metabolism in Alzheimer´s disease. *Free Radic. Biol. Med*. **32**, 1061-1070.

Gibson, G., Martins, R., Blass, J. and Gandy, S. (1996). Atered oxidation and signal transduction systems in fobroblasts from Alzheimer´s patients. *Life Sci.* **59**, 477-489.

Gibson, G. E., Park, L. C., Sheu, K. F., Blass, J. P. and Calingasan, N. Y. (2000). The alpha-ketoglutarate dehydrogenase complex in neurodegeneration. *Neurochem. Int.* **36**, 97-112.

Glenner, G. G. and Wong, C. W. (1984). Alzheimer disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem. Biophys. Res. Commun.* **120**, 885-890.

Goate, A., Chartier-Harlin, M. C., Mullan, M., Brown, J., Crawford, F., Fidani, L., *et al.* (1991). Segregation of a missence mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature* **349**, 704-706.

Goedert, M. (1987). Neuronal localization of amyloid ? protein precursor mRNA in normal human brain and in Alzheimer´s disease. *EMBO J.* **6**, 3627-3632.

Goedert, M., Spillantini, M. G., Cairns, N. J. and Crowther, R. A. (1992). Tau proteins of Alzheimer paired helical filaments: abnormal phosphorylation of all six brain isoforms. *Neuron* **8**, 159-168.

Gong, C. X., Lidsky, T., Wegiel, J., Zuck, L., Grundke-Iqbal, I. and Iqbal, K. (2000). Phosphorylation of microtubule-associated protein tau is regulated by protein phosphatase 2A in mammalian brain. Implications for neurofibrillary degeneration in Alzheimer's disease. *J. Biol. Chem.* **275,** 5535-5544.

Gowing, E., Roher, A. E., Woods, A. S., Cotter, R. J., Chaney, M., Little, S. P. and Ball, M. J. (1994). Chemical characterization of A?17-42 peptide, a component of difuse amyloid deposits of Alzheimer´s disease. *J. Biol. Chem*. **269**, 10987-10990.

Grundke-Iqbal, I., Iqbal, K., Tung, Y. C., Quinlan, M., Wisniewski, H. M. and Binder, L. I. (1986). Abnormal phosphorylation of the microtubule-associated protein tau in Alzheimer cytosKeletal pathology. *Proc. Natl. Acad. Sci. USA* **83**, 4913-4917.

Grütter, M. G. (2000). Caspases: key players in programmed cell death. *Current Opinion Struct. Biol.* **10**, 649-655.

Haas, J., Storch-Hagenlocher, B., Beissmann, A. and Wildemann, B. (2002). Inducible nitric oxide syntethase and argininosuccinate synthetase: co-induction in brain tissue of patients with Alzheimer´s dementia and following stimulation with beta-amyloid 1-42 in vitro. *Neurosci. Lett.* **322**, 121-125.

Haass, C., Schlossmacher, M. G., Hung, A. Y., Vigo-Pelfrey, C., Mellon, A., Ostaszewski, B. L. *et al.* (1992). Amyloid ?-peptides produced by cultured cells during normal metabolism. *Nature* **359**, 322-325.

Haass, C., Lemere, C. A., Capell, A., Citron, M., Seubert, P., Schenk, D., Lannfelt, L. and Selkoe, D. J. (1995). The Swedish mutation causes early-onset Alzheimer´s disease by ?-secretase cleavage within secretory pathway. *Nature Med*. **1**, 1291-1296.

Harada, J. and Sugimoto, M., (1999). Activation of caspase-3 in beta-amyloid induced apoptosis of cultured rat cortical neurons. *Brain Res.* **842**, 311-323.

Hardy, J. and Allsop, D. (1991). Amyloid deposition as the central event in aetiology of Alzheimer's disease. *Trends Pharmacol. Sci.* **12**, 383-388.

Hedin, H. L. and Fowler, C. J. (200). Effects of sodium azide on the secretion of soluble amyloid-beta precursor protein and the accumulation of beta-amyloid(1-40) in cultured chick neurons. *Neurosci. Lett.* **288**, 203-206.

Herceg, Z. and Wang, Z. (1999). Failure of poly(ADP-ribose) polymerase cleavage by caspases leads to induction of necrosis and enhanced apoptosis. *Mol. Cell. Biol.* **19**, 5124-5133.

Holtzman, D. M. (2001). Role of ApoE/Abeta interactions in the pathogenesis of Alzheimer´s disease and cerebral amyloid angiopathy. *J. Mol. Neurosci.* **17**, 147-155.

Hölscher, C. (1998). Possible causes of Alzheimer´s disease: Amyloid fragments, free radicals, and calcium homeostasis. *Neurobiol. Dis.* **5**, 129-141.

Hu, J., Akama, k. T., Krafft, G. A., Chromy, B. A. and Van Eldik, L. J. (1998). Amyloid-beta peptide activates cultured astrocytes: morphological alterations, cytokine induction and nitric oxide release. *Brain Res.* **785**, 195-206.

Huang, X., Atwood, C. S., Hartshorn, M. A., Multhaup, G., Goldstein, L. E., Scarpa, R. C., Cuajungco, M. P., Gray, D. N., Lim, J., Moir, R. D., Tanzi, R. E. and Bush, A. I. (1999). The Abeta peptide of Alzheimer´s disease directly produces hydrogen peroxide through metal ion reduction. *Biochemistry* **38**, 7609-16.

Hung, A. Y., and Selkoe, D. J. (1994). Selective ectodomain phosphorylation and regulated cleavage of beta-amyloid precursor protein. *EMBO J.* **13,** 534-542.

Huse, J. T., Pijack, D. S., Leslie, G. J., Lee, V. M.-Y., and Doms, R. W. (2000). Maturation and endosomal targeting of ?-site amyloid precursor protein-cleaving enzyme. J*. Biol. Chem.* **275**, 33729-33737.

Iwatsubo, T., Okada, A., Susuki, N., Mizusawa, H., Nukina, N. and Ihara, Y. (1994). Visualization of A beta 42 (43) and A beta 40 in senile plaques with end-specific A beta monoclonals: evidence that an initially deposited species is A beta 42. *Neuron* **13**, 45-53.

Jacobsen, J. S., Spruyt, M. A., Brown, A. M., Sahasrabudhe, S. R., Blume, A. J., Vitek, M. P., Muenkel, H. A. and Sonnenberg-Reines, J. (1994). The release of Alzheimer's disease beta amyloid peptide is reduced by phorbol treatment. *J. Biol. Chem.* **269,** 8376**-** 8382.

Johnson, D. A., Gautsch, J. W., Sportsman, J. R. and Elder, J. H. (1984). Improved technique utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. *Gene Anal. Tech.*, 1; 3.

Johnson, G., Refolo, L. M. and Wallace, W. (1993). Heat-shocked neuronal PC12 cells reveal Alzheimer's disease-associated alterations in amyloid precursor protein and tau. *Ann. NY Acad. Sci.* **695**, 194-197.

Kakimura, J. I., Kitamura, Y., Takata, K., Umeki, M., Suzuki, S., Shibagaki, K., Taniguchi, T., Nomura, Y., Gebicke-Haerter, P. J., Smith, M. A., Perry, G. and Shimohama, S. (2002). Microglial activation and amyloid-beta clearance induced by exogenous heat-shock proteins. *FASEB. J.* **16**, 601-603.

Kang, J., Lemaire, H. G., Unterbeck, A., Salbaum, J. M., Master, C. L., Grzeschik, K. H., Multhaup, G., Beyreuther, K. and Muller-Hill, B. (1987). The precursor of Alzheimer´s disease amyloid A4 protein resembles a cell surface receptor. *Nature* **325**, 733-736.

Kienlen-Campard, P., Miolet, S., Tasiaux, B. and Octave, J. N. (2002). Intracellular amyloid-beta 1-42, but not extracellular soluble amyloid-beta peptides, induces neuronal apoptosis. *J. Biol. Chem.* **277**, 15666-15670.

Kim, T. W., Pettingell, W. H., Jung, Y. K., Kovacs, D. M. and Tanzi, R. E. (1997). Alternative cleavage of Alzheimer-associated presenilins during apoptosis by a caspase-3 family protease. *Science* **277**, 373-376.

Kish, S. J., Bergeron, C., Rajput, A., Dozic, S., Mastrogiacomo, F., Chang, L. J., Wilson, J. M., DiStefano, L. M. and Nobrega, J. N. (1992). Brain cytochrome oxidase in Alzheimer´s disease. *J. Neurochem*. **59**, 776-779.

Kosik, K. S. (1993). Alzheimer´s disease: a cell biological perspective, *Science* **256**, 780-783.

Kosik, K. S., Qiu, W. Q., and Greenberg, S. (1996). Cellular signalling pathways and cytoskeletal organization. *Ann. NY Acad. Sci.* **777**, 114-120.

Kowall, N.W., Mckee, A. C., Yanker, B. A. and Beal, M. F. (1992). In vivo neurotoxicity of beta-amyloid [beta(1-40)] and the beta(25-35) fragment. *Neurobiol. Aging* **13**, 537-42.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**; 680-685.

Lauderback, C. M., Hackett, J. M., Huang, F. F., Keller, J. N., Szweda, L. I., Markesbery, W. R. and Butterfield, D. A (2001). The glial glutamate transporter, GLT-1, is oxidatively modified by 4-hidroxy-2-nonenal in the Alzheimer´s disease brain: the role of A?1-42. *J. Neurochem.* **78**, 413-416.

Lauderback, C. M., Kanski, J., Hackett, J. M., Maeda, N., Kindy, M. S. and Butterfield, D. A. (2002). Apolipoprotein E modulates Alzheimer A?(1-42)-induced oxidative damage to synaptosomes in an allele-specific manner. Brain Res. **924**, 90-97.

LeBlanc, A. C. (1995). Increased production of 4KDa amyloid beta peptide in serum deprived human primary neuron cultures: possible involvement of apoptosis. *J. Neurosci.* **15**, 7837-7846.

LeBlanc, A. C., Chen, H., Autilio-Gambetti, L. and Gambetti, P. (1991). Differential APP gene expression in neonatal rat cerebral cortex, meninges and primary astroglial, neuronal and microglial cell. *FEBS Lett*. **292**, 171-178.

Leutz, S., Steiner, B., Marques, C. A., Haass, C., Muller, W. E. and Eckert, A. (2002). Reduction of trophic support enhances apoptosis in PC12 cells expressing Alzheimer´s APP mutation and sensitizes cells to staurosporine-induced cell death. *J. Mol. Neurosci.* **18**, 189-201.

Lyras, L., Cairns, N. J., Jenner, A., Jenner, P. and Halliwell, B. (1997). An assessment of oxidative damage to proteins, lipids, DNA in brain from patients with Alzheimer´s disease. *J. Neurochem.* **68**, 2061-2069.

Loetscher, H., Deuschle, U., Brockhaus, M., Reinhardt, D., Nelboeck, P., Mous, J., Grunberg, J., Haass, C. and Jacobsen, H. (1997). Presenilins are processed by caspase-type proteases. *J. Biochem. Chem*. **272**, 20655-20659.

Lorenzo, A. and Yankner, B. A. (1994). Beta-amyloid neurotoxicity requires fibril formation and is inhibited by congo red. *Proc. Natl. Acad. Sci. USA* **91**, 12243-2247.

Love, S., Barber, R. and Wilcock, G. K. (1999). Increased poly(ADP-ribosyl)ation of nuclear proteins in Alzheimer´s disease. *Brain* **122**, 247-253.

Lukiw, W. J. and Bazan, N. G. (2000). Neuroinflammatory signalling upregulation in Alzheimer´s disease. *Neurochem. Res.* **25**, 1173-1184.

McFarlane, I., Georgopoulou. N., Coughlan, C. M., Gillian, A. M. and Breen, K. C. (1999). The role of the protein glycosylation state in the control of cellular transport of the amyloid beta precursor protein. *Neurosc.* **90**,15-25

Markesbery, W. R. (1997). Oxidative stress hypothesis in Alzheimer´s disease. Free *Radic. Biol. Med.* **23**, 134-147.

Markesbery, W. R. and Carney, J. M. (1999). Oxidative alterations in Alzheimer´s disease. *Brain Pathol.* **9**, 133-146.

Mattson, M. P. (1999). Impairment of membrane transport and signal transduction systems by amyloidogenic proteins. *Methods Enzymol*. **309**, 733-746.

Mattson, M. P., Fu, W., Waeg, G., Uchida, K. (1997). 4-hydroxynonenal, a product of lipid peroxidation, inhibits dephosphorylation of microtubule-associated protein tau. *Neuroreport* **8**, 2275-2281.

Mattson, M. P., Partin, J. and Begley, J. G. (1998). Amyloid beta-peptide induces apoptosis-related events in synapses and dendrites. *Brain Res*. **807**(1-2), 167-176.

Mills, J., Laurent Charest, D., Lam, F., Beyreuther, K., Ida, N., Pelech, S. L. and Reiner, P. B. (1997). Regulation of amyloid precursor protein catabolism involves the mitogen-activated protein kinase signal transduction pathway. *J. Neurosci.* **17**, 9415-9422.

Mills, J. and Reiner, P. B. (1999). Short review: Regulation of Amyloid Precursor Protein Cleavage. *J. Neurochem.* **72**, 443-460.

Misonou, H., Morishima-Kawashima, M. and Ihara, Y. (2000). Oxidative stress induces intracellular accumulation of amyloid beta-protein (Abeta) in human neuroblastome cells. *Biochemistry* **39**, 6951-6959.

Mosser, D. D., Caron, A. W., Bourget, L., Denis-Larose, C. and Massie, B. (1997). Role of the human heat-shock protein hsp70 in protection against stress-induced apoptosis. *Mol. Cell. Biol.* **17**, 5317-5327.

Mossman, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. *J. Immunol. Methods* **65**; 55-63.

Muchowski, P. J., Schaffar, G., Sittler, A., Wanker, E. E., Hayer-Hartl, M. K. and Hartl, F. U. (2000). Hsp70 and hsp40 chaperones can inhibit self assembly of polyglutamine proteins into amyloid-like fibrils. *Proc. Natl. Acad. Sci. USA* **97**, 7841-7846.

Mullan, M., Crawford, F., Axelman, K., Houlden, H., Lilius, L., Winblad, B. and Lannfelt, L. (1992). A pathogenic mutation for probable Alzheimer´s disease in the APP gene at N-terminus of ?-amyloid. *Nat. Genet*. **1**, 345-347.

Murrell, J., Farlow, M., Ghetti, B., and Benson, M. D. (1991). A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease. *Science* **254**, 97-99.

Mutisya, E. M., Bowling, A. C. and Beal, M. F. (1994). Cortical cytochrome oxidase activity is reduced in Alzheimer´s disease. *J. Neurochem*. **63**, 2179-2184.

Nitsch, R. M. and Growdon, J. H. (1994). Role of neurotransmission in the regulation of amyloid ?-protein precursor processing. *Biochem. Pharmacol*. **47**, 1275-1284.

Nitsch, R. M., Deng, M., Growdon, J. H. and Wurtman, R. J. (1996). Serotonin 5-HT2a and 5-HT2c receptors stimulate amyloid precursor protein ectodomain secretion. *J. Biol. Chem.* **271**, 4188-4194.

Nitsch, R. M., Slack, B. E., Farber, S. A., Borghesani, P. R., Schulz, J. G., Kim, C., Felder, C. C., Growdon, J. H. and Wurtman, R. J. (1994). Receptor-coupled amyloid precursor protein processing. *Ann. NY Acad. Sci.* **881**, 122-127.

Nguyen, T. T. and Amara, S. G. (1996). N-linked oligosaccharides are required for cell surface expression of the norepinephrine transporter but do not influence substrate or inhibitor recognition. *J. Neurochem.* **67**, 645-655.

Nunomura, A., Perry, G., Aliev, G., Hirai, K., Takeda, A., Balraj, E. K., Jones, P. K:, Ghanbari, H., wataya, T., Shimohama, S. *et al*. (2001). Oxidative damage is the earliest event in Alzheimer disease. *J. Neuropathol. Exp. Neurol*. **60**, 759-767.

Oishi, M., Nairn, A. C., Czernik, A. J., Lim, G. S., Isohara, T., Gandy, S. E., Greengard, P. and Suzuki, T. (1997). The cytoplasmic domain of Alzheimer´s amyloid precursor protein is phosphorylated at Thr654, Ser655 and Thr668 in adult rat brain and cultured cells. *Mol. Med*. **3**, 111-123.

Olivieri, G., Baysang, G., Meier, F., Muller-Spahn, F., Stahelin, H. B., Brockhaus, M. and Brack, C. (2001). N-acetyl-L-cysteine protects SHSY5Y neuroblastome cells from oxidative stress and cell cytotoxicity: effects on beta-amyloid secretion and tau phosphorylation. *J. Neurochem.* **76**, 224-233.

Ouimet, C. C., da Cruz e Silva, E. F. and Greengard, P. (1995). The alpha and gamma 1 isoforms of protein phosphatase 1 are highly and specifically concentrated in dendritic spines. *Proc. Natl. Acad. Sci. USA* **92**, 3396-3400.

Pahlson, P. and Spitalnik, S. L. (1996). The role of glycosilation in synthesis and secretion of beta-amyloid precursor protein by Chinese hamster ovary cells. *Arch. Biochem. Biophys. Res. Comm.* **189**, 1667-1673.

Parker, W. D. Jr., Mahr, N. J., Filley, C. M., Parks, J. K., Hughes, D., Young, D. A. and Cullum, C. M. (1994). Reduced platelet cytochrome c oxidase activity in Alzheimer´s disease. *Neurology* **44**, 1086-1090.

Parks, J. K., Smith, T. S., Trimmer, P. A., Jr Bennett, J. P. and Jr Parker, W. D. (2001). Neurotoxic A? peptides increase oxidative stress in vivo through NMDA-receptor and nitric-oxide-synthase mechanisms, and inhibit complex IV activity and induce a mitochondrial permeability transition in vitro. *J. Neurochem*. **76**, 1050-1056.

Pellegrini, L., Passer, B. J., Tabaton, M., Ganjei, J. K. and D´Adamio, L. (1999). Alternative, non-secretase processing of Alzheimer´s beta-amyloid precursor protein during apoptosis by caspase-6 and –8. *J. Biol. Chem.* **274**, 21011-21016.

Perry, G., Cash, A. D. and Smith, M. A. (2002). Alzheimer Disease and Oxidative Stress. *J. Biomed. Biotechnol*. **2**, 120-123.

Pike, C. J., Cummings, B. J. and Cotman, C. W. (1992). Beta-Amyloid induces neuritic dystrophy in vitro: similarities with Alzheimer pathology. *Neuroreport* **3**, 769-772.

Pike, C. J., Overman, M. J. and Cotman, C. W. (1995). Amino-terminal deletions enhance aggregation of ?-amyloid peptides in vitro. *J. Biol. Chem*. **270**, 23895-23898.

Prasad, K. N., Cole, W. C. and Prasad, K. C. (2002). Risk factors for Alzheimer´s disease: role of multiple antioxidants, non-steroidal anti-inflammatory and cholinergic agents alone or in combination in prevention and treatment. *J. American Coll. Nutri.* **21**, 506-522.

Pratico, D., Uryu, K., Leight, S., Trojanoswki, J. Q. and Lee, V. M. (2001). Increased lipid peroxidation precedes amyloi plaque formation in an animal model of Alzheimer amyloidosis. *J. Neurosci*. **21**, 4183-4137.

Price, D. L., Borchelt, D. R. and Sisoda, S. S (1993). Alzheimer disease and the prion disorders amyloid ?-protein and prion protein amyloidoses. Proc. *Natl. Acad. Sci. USA* **90**, 6381-6384.

Renkawek, K., Voorter, C. E., Bosman, G. J., van Workum, F. P. and de Jong, W. W. (1994). Expression of alpha B-crystallin in Alzheimer´s disease. *Acta Neuropathol. (Berl)* **87**, 155-160.

Richard, F. and Amouyel, P. (2001). Genetic susceptibility factors for Alzheimer's Disease. *Eur. J. Pharmacol.* **412**, 1-12.

Tanzi, R. E., Kovacs, D. M., Kim, Tae-Wan, Moir, R. D., Guenette, S. Y. and Wasco, W. (1996). The gene defects responsible for familial Alzheimer´s disease. *Neurobiol. Dis*. **3**, 159-168.

Sabo, S. L., Lanier, L. M., Ikin, A. F., Khorkova, O., Sahasrabudhe, S., Greengard, P. and Buxbaum, J. D. (1999). Regulation of beta-amyloid secretion by FE65, an amyloid protein precursor-binding protein. J*. Biol. Chem.* **274**, 7952-7957.

Savage, M. J., Trusko, S. P., Howland, D. S., Pinsker, L. R., Mistretta, S., Reaume, A. G., Greenberg, B. D., Siman, R. and Scott, R. W. (1998). Turnover of amyloid ?-protein in mouse brain and acute reduction of its level by phorbol ester. *J. Neurosci*. **18**, 1743-1752.

Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T. D., Hardy, J., Hutton, M., Kukull, W., Larson, E. *et al*., (1996). Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer´s disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer´s disease. *Nat. Med*. **2**, 864-870.

Selkoe, D. J. (1994). Cell biology of the amyloid beta-protein precursor and the mechanism of Alzheimer´s disease. *Annu. Rev. Cell Biol*. **10**, 373-403.

Selkoe, D. J. (1997). Alzheimer´s disease: Genotypes, phenotype, and treatments. *Science* **275**, 630-632.

Selkoe, D. J. and Kopan, R. (2003). Notch and presenilin: regulated intramembrane proteolysis links development and degeneration. *Annu. Rev. Neurosci.* [epub ahead of print].

Selkoe, D. J., Yamazaki, M., Citron, M., Podlisny, M. B., Koo, E. H., Teplow, D. B. and Haass, C. (1996). The role of APP processing and trafficking pathways in the formation of amyloid ?-protein. *Ann. NY Acad. Sci.* **777**, 57-64.

Shinohara, H., Inaguma, Y., Goto, S., Inagaki, T. and Kato, K. (1993). Alpha B crystallin and HSP28 are enhanced in the cerebral cortex of patients with Alzheimer´s disease. *J. Neurol. Sci*. **119**, 155-160.

Smith, M. A., Harris, P. L. R., Sayre, L. M. and Perry, G. (1997). Iron accumulation in Alzheimer disease is a source of redox-generated free radicals. *Proc. Natl Acad Sci USA*. **94**, 9866-9868.

Smith, M. A., Rottkamp, C. A., Nunomura, A., Raina, A. K. and Perry, G. (2000). Oxidative stress in Alzheimer´s disease. *Biochim. Biophys. Acta* **1502**, 139-144.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76-85.

Shoji, M., Golde, T. E., Ghiso, J., Cheung, T. T., Estus, S., Shaffer, L. M., *et al.* (1992). Production of the Alzheimer amyloid ? protein by normal proteolytic processing. *Science* **258**, 126-129.

SOti, C. and Csermely, P. (2000). Molecular chaperones and the aging process. *Biogerontology* **1**, 225-233.

Soto, C. (1999). Alzheimer's and prion disease as disorders of protein conformation: Implications for the design of novel therapeutic approaches. *J. Mol. Med.* **77**, 412-418.

Strosznajder, J. B., J sko, H. and Strosznajder R. P. (2000). Effect of amyloid beta peptide on poly(ADP-ribose) polymerase activity in adult and aged rat hippocampus. *Acta. Biochimi. Pol.* **47**, 847-854.

Su, J. H., Anderson, A. J., Cummings, B. J. and Cotman, C. W. (1994). Immunohistochemical evidence for apoptosis in Alzheimer´s disease. *Neuroreport*, **5**, 2529-2533.

Suzuki, T., Oishi, M., Marshak, D. R., Czernik, A. J., Nairn, A. C. and Greengrad, P. (1994). Cell cycle-dependent regulation of the phosphorylation and metabolism of the Alzheimer amyloid precursor protein. *EMBO J.* **13**, 1114-1122.

Tan, S., Maher, P. and Schubert, D. (1997). The role of protein phosphorylation in beta amyloid toxicity. *Brain Res.* **765**, 159-163.

Tanzi, R. E., Gusella, J. F., Watkins, P. C., Bruns, G. A., St. George-Hyslop, P., Van Keuren, M. L., Patterson, D., Pagan, S., Kurnit, D. M. and Neve, R. L. (1987). Amyloid ? protein gene: cDNA, mRNA distribution and genetic linkage near Alzheimer locus. *Science* **235**, 880-884.

Tanzi, E. R., Kovacs, D. M., Kim, T., Moir, R. D., Guenette, S. Y. and Wasco, W. (1996). The gene defects responsible for familial Alzheimer´s disease. *Neurobiol. Dis.* **3**, 159-168.

Tanzi, R. E., Vaula, G., Romano, D. M., Mortilla, M., Huang, T. L., Tupler, R. G., Wasco, W., Hyman, B. T., Haines, J. L., Jenkins, B. J. *et al.* (1992). Assessment of amyloid beta-protein precursor gene mutations in a large set of familial and sporadic Alzheimer disease cases. *Am. J. Hum. Genet*. **51**, 273-82.

Tewari, M., Quan, L. T., O´Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salvesen, G. S. and Dixit, V. M. (1995). Yama/CPP32B, a mammalian homolog of CED-3, is a Crm-Ainhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* **81**, 801-809.

Troncoso, J. C., Sukhov, R. R., Kawas, C. H. and Koliatsos, V. E. (1996). In situ labeling of dying cortical neurons in normal aging and in Alzheimer´s disease: correlations with senile plaques and disease progression. *J. Neuropathol. Exp. Neurol.* **55**, 1134-1142.

Varadarajan, S., Kanski, J., Aksenova, M., Lauderback, C. and Butterfield, D. A. (2001). Different mechanisms of oxidative stress and neurotoxicity for Alzheimer´s abeta (1-42) and abeta (25-35). *J. Am. Chem. Soc.* **123**, 5625-5631.

Varadarajan, S., Yatin, S., Aksenova, M. and Butterfield, D. A. (2000). Review: Alzheimer´s amyloid beta-peptide-associated free radical oxidative stress and neurotoxicity. *J. Struct.Biol.* **130**, 184-208.

Vassar, R., Bennet, B. D., Babu-Khan, S., Kahn, S., Mendiaz, E. A., Denis, P., Teplow, D. B., Ross, S., Amarante, P., Loeloff, R., Luo, Y., Fisher, S., Fuller, J., Edenson, S., Lile, J., Jarosinski, M. A., Biere, A. L., Curran, E., Burgess, T., Louis, J., Collins, F., Treanor, J., Rogers, G. and Citron, M. (1999). Beta-secretase cleavage of Alzheimer´s amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* **286**, 735-741.

Vickers, J. C., Dickson, T. C., Adlard, P. A., Saunders, H. L., King, C. E. and McCormack, G. (2000). The cause of neuronal degeneration in Alzheimer´s disease. *Prog. Neurobiol.* **60**(2), 139-165.

Wang, J., Dickson, D. W., Trojanowski, J. Q., and Lee, V. M. (1999). The levels of soluble versus insoluble brain Abeta distinguish Alzheimer´s disease from normal and pathological aging. *Exp. Neurol*. **158**, 328-337.

Weidemann, A., Konig, G., Bunke, D., Fisher, P., Salbaum, J. M., Masters, C. L. and Beyreuther, k. (1989). Identification, biogenesis, and localization of precursors of Alzheimer´s disease A4 amyloid protein. *Cell* **57**, 115-126.

Whitehead, T. P., Kricha, L. J., Carter, T. J. and Thorpe, G. H. (1979). Analytical luminescence: its potential in the clinical laboratory. *Clin. Chem*. **25**, 1531-1546.

Williams, B. R. (2001). Geriatric dementias. In: Koda Kimble, M. A., Young, L. Y. Eds. *Applied therapeutics: the clinical use of drugs*, 7th edn. Lippincott: Williams & Wilkins, 98.1-98.9.

Xu, H., Jeitner, T., Zhang, H. and Gibson, G. E. (2001). *J. Neurochm.* **78**S, 32.

Yankner, B. A., Caceres, A. and Duffy, L. K. (1990). Nerve growth factor potentiates the neurotoxicity of beta amyloid. *Proc. Natl. Acad. Sci. USA* **87**, 9020-9023.

Yatin, S. M., Yatin, M., Varadarajan, S., Ain, K. B: and Butterfield, D. A. (2001). Role of spermine in amyloid ?-peptide-associated free radical-induced neurotoxicity. *J. Neurosci. Res.* **63**, 395-401.

Yatin, S. M., Varadaranjan, S., Link, C. D. and Butterfield, D. A. (1999). In vitro and in vivo oxidative stress associated with Alzheimer´s amyloid beta-peptide (1-42). *Neurobiol. Aging* **20**, 325-330.

Yoo, B. C., Kim, S. H., Cairns, N., Fountoulakis, M. and Lubec, G. (2001). Deranged expression of molecular chaperones in brains of patints with Alzheimer´s disease. *Biochem. Biophys. Res. Commun.* **280**, 249-258.

Yoo, B. C., Seidl, R., Cairns, N. and Lubec, G. (1999). Heat-shock protein 70 levels in brain of patients with Down syndrome and Alzheimer´s disease. *J. Neural. Transm. Suppl.* **57**, 315-322.

Zhang, Y., McLaughlin, R., Goodyer, C. and Leblanc, A. (2002). Selective cytotoxicity of intracellular amyloid beta peptide 1-42 through p53 and Bax in cultured primary human neurons. *J. Cell Biol.* **156**, 519-529.

7. ANNEXES

Bellow is listed the equipment used and composition of buffers for the different techniques applied.

7.1 For cell culture and experimental models

Equipment

Hera cell CO_2 incubator (Heraeus)
Safety cabinet Hera safe (Heraeus)
Inverted optical microscope (LEICA)
Hemacytometer (Sigma)
Sonicator U200S (IKA)
Bath SBB6 (Grant)

Reagents

Dulbecco's modified Eagle's medium (DMEM; Sigma)
RPMI 1640(GIBCO BRL)
Foetal bovine serum (GIBCO BRL)
Horse serum (GIBCO BRL)
Antimycotic-antibiotic solution (GIBCO BRL)
Trypsin-EDTA (GIBCO BRL)
Poly-L-ornithine (Sigma)
$NaHCO_3$ (Sigma)
Abeta 1-40 and Abeta 25-35 (Sigma)
2-deoxyglucose (2DG) (Calbiochem)
Sodium azide (NaN_3) (Merck)
Phosphate buffer saline (PBS) (Pierce)
AO (Calbiochem)
PMA (Calbiochem)

7.2 For measurement of cell viability

Equipment

Spectrophotometer Cary 50 (Varian)

Reagents

MTT [3-(4,5dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma)
Acidic isopropanol (0.04 M HCl in absolute isopropanol)

7.3 For protein content determination

Equipment

Spectrophotometer Cary 50 (Varian)

Reagents

BCA assay kit (Pierce, Rockford, IL)

Bovine serum albumin (BSA) (Pierce)

Working reagent (Reagent A: sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0.2 N sodium hydroxide; reagent B: 4% cupric sulfate).

7.4 For SDS-PAGE

Equipment

Electrophoresis system (Hoefer SE600 vertical unit)

Electrophoresis power supply EPS 1000 (Pharmacia Biotec)

Reagents

Acrylamide stock mixture (30% acrylamide, 0.8% N,N'-methylenediacrylamide)

Stacking gel (H_20, 30% acrylamide mixture, 4x stacking gel buffer (0.63 M Tris, pH 6.8), 10% SDS, 10% ammonium persulfate, TEMED)

Resolving gel (H_20, 30% acrylamide mixture, 4x resolving gel buffer (1.5 M Tris-HCl, 0.014 M SDS, pH 8.8), 10% ammonium persulfate, TEMED)

10x Running Buffer (0.25 M Tris-HCl, 1.92 M Glycine, pH 8.3, 0.1% SDS)

4? SDS gel-loading buffer (250 mM Tris-HCl pH 6.8, 8% SDS, 0.01% bromophenol blue, 20% 2-mercaptoethanol, 40% glycerol)

Kaleidoscope/prestained SDS-PAGE standards (Bio-Rad)

7.5 For Western blotting

Equipment

Transphor Electrophoresis unit (Hoefer TE 42)

Electrophoresis power supply EPS 1000 (Amersham Pharmacia Biotech)

Reagents

Transfer buffer (0.025 M Tris base, 0.19 M Glycine, 20% methanol, pH 8.3)

7.6 For immunodetection of proteins

Reagents

10x Tris-buffered saline (TBS) (0.1M Tris, 11.5M NaCl, pH 8.0)
10? Tris-buffered saline Tween (TBS-T) stock (0.1M Tris, 11.5M NaCl, 0.5% Tween 20, pH 8.0)
Blocking solution (5% nonfat dried milk in 1x TBS-T)
ECL or ECL Plus Kits (Amersham Biosciences)
Developer and fixer solution (Sigma)
Bromocloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (Promega)
Alkaline phosphatase buffer (100mM NaCl, 100mM Tris (pH 9.5), 5mM $MgCl_2$)
Substrate solution (66?l of NBT in 10ml of alkaline phosphatase buffer, mix and add 33?l of BCIP)
Stop solution (20mM Tris, pH 9.5, 5 mM EDTA)

7.7 For statistical analysis

Equipment

GS-710 calibrated imaging densitometer (Bio-Rad)